Dynamic Modulation of Interendothelial Gap Junctional Communication by 11,12-Epoxyeicosatrienoic Acid

Rüdiger Popp,* Ralf P. Brandes,* Gregor Ott, Rudi Busse, Ingrid Fleming

Abstract—Functional gap junctional communication between vascular cells has been implicated in ascending dilatation and the cytochrome P-450 (CYP) inhibitor–sensitive and NO- and prostacyclin-independent dilatation of many vascular beds. Here, we assessed the mechanisms by which the epoxyeicosatrienoic acids (EETs) generated by a CYP 2C enzyme control interendothelial gap junctional communication. In CYP 2C–expressing porcine coronary endothelial cells, bradykinin, which enhances EET formation, elicited a biphasic effect on the electrical coupling and transfer of Lucifer yellow between endothelial cells, consisting of a transient increase in coupling followed by a sustained uncoupling. The initial phase was sensitive to the CYP 2C9 inhibitor sulfaphenazole and the protein kinase A (PKA) inhibitors Rp-cAMPS and KT5720 and could be mimicked by forskolin and caged cAMP as well as by the PKA activators 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole 3',5'-cyclic monophosphorothioate sodium salt and Sp-cAMPS. Gap junction uncoupling in bradykinin-stimulated porcine coronary endothelial cells was prevented by inhibiting the activation of extracellular signal–regulated kinase (ERK)1/2. In human endothelial cells, which express little CYP 2C, bradykinin elicited only an ERK1/2-mediated inhibition of intercellular communication. The CYP 2C9 product, 11,12-EET, also exerted a dual effect on the electrical and dye coupling of human endothelial cells, which was sensitive to PKA inhibition. These results demonstrate that an agonist-activated CYP-dependent pathway as well as 11,12-EET can positively regulate interendothelial gap junctional communication, most probably via the activation of PKA, an effect that is curtailed by the subsequent activation of ERK1/2.

In large arteries and veins, gap junctions are known to be essential for the propagation of electrical signals between vascular smooth muscle cells, whereas in the microcirculation they appear to be involved in the phenomena of ascending dilation and endothelium-derived hyperpolarization factor (EDHF)-mediated relaxation (see review1). Myoendothelial gap junctions have been identified in numerous vascular beds, particularly in small arteries and terminal arterioles,2 and it is assumed that the selective gating of such gap junctions plays an integral role in endothelium-dependent and in NO- and prostacyclin (PGI2)-independent relaxation.3–6 However, there has been no convincing demonstration of a direct link between dynamic alterations in myoendothelial gap junctions and relaxation/vasodilatation.

Interendothelial, rather than myoendothelial, cell communication has recently been proposed to be the pathway by which hyperpolarization and vasodilatation are conducted along agonist-stimulated resistance vessels.7 In mice and hamsters,8–10 this phenomenon has been linked to a cytochrome P-450 (CYP)-dependent process that demonstrates characteristics similar to the NO/PGL2-independent relaxation described in porcine epicardial arteries11 and hamster resistance-sized arteries.12 The latter endothelium-dependent relaxation is thought to be determined by the generation of epoxyeicosatrienoic acids (EETs), such as 11,12-EET, and by an endothelial CYP epoxygenase belonging to the CYP 2C family.

The aim of the present study was to determine whether or not interfering with the generation and/or action of 11,12-EET affects interendothelial gap junctional communication. To this end, two endothelial cell types were used: (1) porcine coronary endothelial cells, which express CYP 2C protein and which generate EETs even after 2 to 3 days in culture,11 and (2) passaged human umbilical vein endothelial cells, which express little or no CYP 2C protein or mRNA.13

Materials and Methods

Materials

Bradykinin was from Bachem Biochemica GmbH; PD 98059 was from Biomol; U0126 and 4,5-dimethoxy-2-nitrobenzyl–caged
cAMP were from Calbiochem-Novabiochem; and 11,12-EET was from Cayman Chemical. The connexin43 (Cx43) monoclonal antibody was from Transduction Laboratories. The phospho–extracellular signal–regulated kinase (ERK)1/2 antibodies (recognizing Thr202/Tyr204) and the ERK1/2 antibodies were from New England Biolabs Inc. The protein kinase A (PKA) inhibitors Rp-cAMPS and KT5720 and the PKA activators Sp-cAMPS and 5,6-dichloro-1-β-D-ribofuranylosibenzimidazole 3’,5’-cyclic monophosphorothioate sodium salt (Sp-5,6-DCl-cBiMPS) were from Alexis Biochemicals. 2’,5’-Dideoxyadenosine (2’,5’-DDA) and all other chemicals were purchased from Sigma Chemical Co.

Cell Culture
Human umbilical vein endothelial cells and porcine coronary artery endothelial cells were isolated and cultured as described. In some experiments, a commercially available kit (NEN Life Science Products) was used to assess intracellular cAMP in cells pretreated with 3-isobutyl-1-methylxanthine (30 μmol/L).

Determination of Gap Junctional Communication by Lucifer Yellow Dye Coupling
Sharp microelectrodes (tip resistance ~50 MΩ) were loaded with the fluorescent tracer Lucifer yellow (4% in 100 mmol/L lithium chloride). Glass coverslips containing a monolayer of endothelial cells were superfused with a modified Tyrode’s solution containing (mmol/L) NaCl 132, KCl 4, CaCl2 1.6, MgCl2 0.98, NaHCO3 20, NaH2PO4 0.36, glucose 10, and CaCl2-EDTA 0.05 and were gassed with 20% O2/5% CO2/75% N2 to yield a PO2 of 140 mm Hg and pH 7.4. The pipette resistance was between 5 and 10 MΩ, and capacitative currents were filtered with a low-pass filter (1 kHz) and digitalized. The area under the transient, which represents the charge accumulated on the membrane condenser, was calculated by using specialized software (Pulse, HEKA-Elektronik). Because these transients cannot be calculated on the basis of a single exponential function, changes in capacitance are expressed relatively to capacitance in unstimulated cells.

Immunoblotting
Cells were lysed in buffer containing Tris/HCl (pH 7.5, 50 mmol/L), NaCl (150 mmol/L), NaF (100 μmol/L), NaPO4 (15 mmol/L), Na2VO4 (2 mmol/L), leupeptin (2 μg/mL), pepstatin A (2 μg/mL), trypsin inhibitor (10 μg/mL), phenylmethylsulfonyl fluoride (44 μg/mL), and Triton X-100 (1% [vol/vol]); they were then left on ice for 10 minutes and centrifuged at 10 000 g for 10 minutes. Proteins in the Triton X-100–soluble and –insoluble fractions were heated with SDS-PAGE sample buffer and separated by SDS-PAGE, as described. Proteins were detected by using their respective antibodies, as described in Results, and were visualized by enhanced chemiluminescence with the use of a commercially available kit (Amersham).

To reprobe Western blots with alternative primary antibodies, the nitrocellulose membranes were incubated at 50°C for 30 minutes in a buffer containing Tris/HCl (67.5 mmol/L, pH 6.8), β-mercaptoethanol (100 mmol/L), and SDS (2%). After extensive washing in buffer containing Tris (50 mmol/L, pH 7.5) and NaCl (200 mmol/L), the filters were incubated in blocking buffer containing BSA (3%) and, subsequently, with the primary antibody.

Statistical Analysis
Data are expressed as mean±SEM, and statistical evaluation was performed by using the Student t test for unpaired data, 1-way ANOVA followed by the Bonferroni t test, or ANOVA for repeated measures where appropriate. Values of P<0.05 were considered statistically significant.

Results
Effect of Bradykinin on Gap Junctional Communication
In coronary artery endothelial cells, bradykinin (10 nmol/L) exerted a biphasic effect on both the electrical coupling (Figure 1A) and transfer of Lucifer yellow (Figure 1B). The response consisted of a rapid increase, which peaked after ~60 seconds, followed by a maintained decrease in coupling. The bradykinin-induced changes in gap junctional communication were unrelated to the generation of either NO or PGI2, inasmuch as neither phase of the response was affected by the inclusion of L-NA and diclofenac (Figure 1C). Moreover, the organic nitrate sodium nitroprusside (1 μmol/L) failed to significantly affect gap junctional communication; the number of Lucifer yellow–labeled cells was 24.1±3.3 in untreated endothelial cells versus 23.6±2.3 and 20.4±3.0 at 1 and 5 minutes, respectively, after the application of sodium nitroprusside (n=9).

However, the transient increases in electrical capacitance and Lucifer yellow transfer (in the presence of L-NA and diclofenac) were abolished by sulfaphenazole (Figure 1C), which inhibits CYP 2C9 activity in coronary endothelial cells. The combination of charybotoxin and apamin, which abrogates NO/PGI2–independent hyperpolarization and relaxation, was without effect (Figure 1C). Neither sulfaphenazole nor the combination of the Ca2+-dependent K+ (KCa) channel...
inhibitors significantly affected dye coupling when given alone (data not shown).

A second receptor-dependent agonist, substance P (1 μmol/L), failed to affect the intracellular transfer of Lucifer yellow. The number of Lucifer yellow–labeled cells was 21.7±1.6 in untreated endothelial cells versus 22.5±3.6 and 18.8±2.8 at 1 and 5 minutes, respectively, after the application of substance P (n=7). The coronary artery endothelial cells that were studied expressed a functional substance P receptor as the agonist induced a pronounced hyperpolarization (34.8±2 mV, n=5).

In cultured human endothelial cells, which express low levels of CYP 2C, bradykinin did not elicit an increase in interendothelial coupling but time-dependently inhibited gap junctional communication assessed by either the changes in capacitance or the intercellular diffusion of Lucifer yellow (data not shown). However, in these cells, the CYP 2C product, 11,12-EET, transiently enhanced the coupling of endothelial cells before uncoupling them (Figure 2A). The number of Lucifer yellow–labeled cells was 16.9±2 in untreated endothelial cells versus 25.2±2.4, 4.2±1.1, and 4.6±0.7 cells at 1, 5, and 10 minutes, respectively, after the application of 1,12-EET (P<0.01, n=5). A similar time course was observed for the 11,12-EET–induced changes in electrical capacitance (Figure 2B). The initial 11,12-EET–induced increase in cell-cell communication was unaffected by either sulfaphenazole or the combination of charybdotoxin and apamin (Figure 2C).

**Effect of Increasing CYP 2C Expression on Gap Junctional Communication**

To further demonstrate the link between CYP 2C and gap junctional communication, we determined the effects of nifedipine on the transfer of Lucifer yellow. As reported previously, pretreatment of porcine coronary arterial and human umbilical vein endothelial cells was associated with an enhanced basal coupling of both endothelial cell types, and the selective CYP 2C9 inhibitor, sulfaphenazole, reversed the effects of nifedipine preincubation on endothelial cell coupling (Figure 3).

**Effect of cAMP on Gap Junctional Communication and Connexin Phosphorylation**

Because cAMP has been suggested to act as a second messenger for 11,12-EET, we assessed the effects of this nucleotide on the interendothelial transfer of Lucifer yellow. The adenylyl cyclase activator forskolin (10 μmol/L) significantly increased dye transfer, and a similar response was observed after the intracellular release of cAMP from a caged compound (Figure 4A). The cAMP-induced increase in endothelial cell coupling was associated with a time-dependent translocation of Cx43 to the Triton X-100–insoluble cell fraction. Both the increase in cell coupling (data not

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**Figure 1.** Bradykinin-induced changes in gap junctional communication. A and B, Time course of the bradykinin (10 nmol/L)–induced changes in electrical coupling (A) and the transfer of Lucifer yellow (B) between porcine coronary artery endothelial cells. Experiments were performed in the absence and presence of L-NA (300 μmol/L) and diclofenac (diclo, 10 μmol/L). C, Pharmacological characterization of the communication-enhancing factor in porcine coronary endothelial cells. Dye (Lucifer yellow) transfer between endothelial cells was assessed in cells pre-treated with solvent, sulfaphenazole (Sulfa, 10 μmol/L), or the combination of charybdotoxin/apamin (CA, both 100 nmol/L) under basal conditions and 60 seconds after the application of bradykinin (100 nmol/L). Experiments were performed in the continuous presence of L-NA (300 μmol/L) and diclo (10 μmol/L), and the results represent the mean±SEM of data obtained in 8 separate experiments. *P<0.05 and **P<0.01 vs control.

**Figure 2.** Time course of the 11,12-EET–induced changes in gap junctional communication between human umbilical vein endothelial cells. A and B, Endothelial cells were stimulated with 11,12-EET (3 μmol/L) for the time shown, and the transfer of Lucifer yellow (A) and the electrical coupling (B) between endothelial cells were assessed. C, Dye transfer between human endothelial cells was assessed in cells pretreated with solvent, Sulfa (10 μmol/L), or CA (both 100 nmol/L) under basal conditions and 60 seconds after the application of 11,12-EET (3 μmol/L). Experiments were performed in the continuous presence of L-NA (300 μmol/L) and diclofenac (10 μmol/L), and the results represent the mean±SEM of data obtained in 8 separate experiments. *P<0.05 and **P<0.01 vs control (CTL).

To further demonstrate the link between CYP 2C and gap junctional communication, we determined the effects of nifedipine on the transfer of Lucifer yellow. As reported previously, pretreatment of porcine coronary arterial and human umbilical vein endothelial cells with nifedipine (0.1 μmol/L, 18 hours) induced a significant increase in CYP 2C RNA expression as well as a significant increase in the generation of 11,12-EET. This increase in CYP expression was associated with an enhanced basal coupling of both endothelial cell types, and the selective CYP 2C9 inhibitor, sulfaphenazole, reversed the effects of nifedipine preincubation on endothelial cell coupling (Figure 3).

The adenylyl cyclase activator forskolin (10 μmol/L) significantly increased dye transfer, and a similar response was observed after the intracellular release of cAMP from a caged compound (Figure 4A). The cAMP-induced increase in endothelial cell coupling was associated with a time-dependent translocation of Cx43 to the Triton X-100–insoluble cell fraction. Both the increase in cell coupling (data not

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Role of cAMP in Increasing Gap Junctional Communication in Bradykinin-Stimulated and 11,12-EET-Stimulated Endothelial Cells

In cultured porcine coronary endothelial cells, bradykinin increased intracellular levels of cAMP, an effect prevented by pretreating cells with sulfinaphenazole. The effect of 11,12-EET on cAMP was less marked, although significant, but was insensitive to the CYP inhibitor (Figure 5A).

To assess the effects of interfering with cAMP generation and the activation of PKA on cell coupling, we determined the effects of the adenylyl cyclase inhibitor 2',5'-DDA, the PKA inhibitors Rp-cAMPS and KT5720, and the PKA activators Sp-cAMPS and Sp-5,6-DDA, nor KT5720 (1 μmol/L) affected the transfer of Lucifer yellow between solvent-treated porcine endothelial cells. However, all three compounds prevented the bradykinin-induced increase in endothelial cell coupling (Figures 5B and 5C; KT5720; data not shown).

In human endothelial cells, KT5720 prevented the increase in coupling elicited by increasing intracellular cAMP and the exogenous application of 11,12-EET (Figure 5D). Rp-cAMPS also prevented the transient increase in endothelial cell coupling in human endothelial cells stimulated with 11,12-EET; 11,12-EET (1 μmol/L, 60 seconds) increased the number of cells coupled from 35.5 ± 7.5 to 62.0 ± 11.0 in the absence of Rp-cAMPS (P<0.05) and from 31.0 ± 3 to 33.4 ± 3.0 in the presence of Rp-cAMPS (n=4).
Role of ERK1/2 in the Uncoupling of Bradykinin-Stimulated and 11,12-EET-Stimulated Endothelial Cells

To elucidate the mechanism by which bradykinin and 11,12-EET induced the maintained uncoupling of endothelial cells, experiments were performed in the absence and presence of sulfaphenazole and charybotoxin/apamin. As shown in Figure 1, cell coupling was decreased below basal levels 10 minutes after the application of bradykinin. This effect did not appear to be related to the activation of CYP 2C, inasmuch as sulfaphenazole did not prevent significant uncoupling (Figure 6A). Moreover, charybotoxin and apamin, which prevent the bradykinin-induced hyperpolarization of endothelial cells, were also without effect, indicating that the changes in coupling were not governed by alterations in membrane potential (Figure 6A). However, pretreating endothelial cells with the mitogen-activated protein kinase kinase (MEK) inhibitors PD 98059 (Figures 6A and 6B) or U0126 (data not shown) to prevent the activation of ERK1/2 completely abolished the bradykinin-induced uncoupling of endothelial cells. In contrast, PD 98059 did not affect the transient increase in endothelial cell coupling observed 1 minute after addition of the agonist (Figure 6B).

Incubation of human endothelial cells with either bradykinin or 11,12-EET for 10 minutes attenuated the intercellular transfer of Lucifer yellow. The extent of inhibition of gap junctional communication elicited by 11,12-EET was concentration dependent, and as in the experiments using bradykinin-stimulated coronary artery endothelial cells, the decrease in the diffusion of Lucifer yellow was prevented by PD 98059 (Figure 6C).

To demonstrate a link between the activation of ERK1/2 and alterations in Cx43 phosphorylation, we compared the time courses of ERK1/2 activation with alterations in the phosphorylation of Cx43 in the Triton-soluble cell fraction. The time course of ERK1/2 phosphorylation after the application of bradykinin was similar in human and porcine endothelial cells and was slightly faster than that observed using 11,12-EET (Figure 7). The phosphorylation of ERK1/2 was paralleled by a shift in the mobility of Cx43 in the SDS-PAGE gel, which has previously been described to reflect Cx43 phosphorylation.

Figure 6. Representative experiment showing the time course of bradykinin (100 nmol/L) or 11,12-EET (1 μmol/L) on the coupling of porcine coronary endothelial cells in the absence and presence of solvent, Sulfa (10 nmol/L, 10 minutes). Experiments were performed in the absence and presence (striped bar) of PD 98059. The results represent the mean±SEM of data obtained in 10 separate experiments. *P<0.05, **P<0.01, and ***P<0.001 vs control.

Figure 7. Western blots showing the effect of the MEK inhibitor U0126 on the bradykinin-induced and 11,12-EET-induced phosphorylation of Cx43 in human umbilical vein endothelial cells. Confluent cultures of endothelial cells were stimulated with bradykinin (100 nmol/L) or 11,12-EET (1 μmol/L) in the absence and presence of U0126 (1 μmol/L) for the times indicated. Triton X-100–soluble cell fractions were subjected to SDS-PAGE, and Cx43 was identified by using a specific antibody. The Cx43 band can be separated into the nonphosphorylated protein (NP) and 2 phosphorylated forms (P1 and P2). To compare the time course of the Cx43 mobility shift with the activation of ERK1/2, each blot was reprobed with a specific antibody recognizing the phosphorylated form of ERK1/2 (pERK1/2) as well as total ERK1/2 protein. Identical results were obtained in 2 additional experiments.

Role of ERK1/2 in mediating the delayed bradykinin-induced and 11,12-EET–induced uncoupling of endothelial cells. A, Capacitance measurements showing the effect of bradykinin (100 mmol/L, 20 minutes) on the coupling of porcine coronary endothelial cells in the absence and presence of solvent, Sulfa (10 nmol/L), CA (both 100 nmol/L), or PD 98059 (50 μmol/L). B, Concentration-dependent inhibition of endothelial cell dye (Lucifer yellow) coupling by 11,12-EET (1 to 10 μmol/L, 10 minutes). C, Concentration-dependent inhibition of endothelial cell dye (Lucifer yellow) coupling by 11,12-EET (1 to 10 μmol/L, 10 minutes). Experiments were performed in the absence and presence of PD 98059. The results represent the mean±SEM of data obtained in 10 separate experiments. *P<0.05, **P<0.01, and ***P<0.001 vs control.

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The activation of ERK1/2 and the Cx43 mobility shifts were prevented in cells pretreated with the MEK inhibitors PD 98059 (data not shown) and U0126 (Figure 7).
Discussion
In the present study, we have demonstrated that a bradykininin-activated CYP-derived metabolite and an exogenously applied 11,12-EET rapidly (within 1 minute) but transiently enhanced the electrical coupling and the transfer of Lucifer yellow between endothelial cells. This initial response was not related to the activation of $K_{Ca}$ channels but was dependent on the generation of cAMP and the activation of PKA. The transient increase in cell coupling was followed by an almost complete inhibition of interendothelial cell communication, which was associated with the activation of ERK1/2.

Despite the fact that the vast majority of connexons in a gap junction plaque are assumed to exist in an open configuration, it is feasible that the induction of a physiological response could elicit an increase in interendothelial communication. Using porcine coronary endothelial cells, which express CYP 2C and generate EETs, we observed that bradykinin induced biphasic alterations in electrical communication and dye transfer within a cell monolayer, consisting of a transient increase in coupling that preceded a maintained uncoupling. In human umbilical vein endothelial cells, which express little CYP 2C, bradykinin did not enhance either electrical or dye coupling but elicited only the delayed phase of uncoupling. However, in these cells, 11,12-EET, which is a product of the metabolism of arachidonic acid by CYP 2C, also enhanced the transfer of Lucifer yellow before uncoupling was observed. Moreover, inducing the expression of CYP 2C in human endothelial cells with the use of nifedipine markedly enhanced endothelial cell coupling in a manner sensitive to the CYP 2C9 inhibitor sulfaphenazole. An enhanced coupling was also observed in cultured porcine aortic endothelial cells stably transfected with CYP 2C9 (authors’ unpublished data, 2002). An additional series of experiments was performed with the use of substance P. This agonist was chosen because, in contrast to bradykinin, it is unable to elicit the activation of CYP 2C or the generation of 11,12-EET and 14,15-EET in coronary artery endothelial cells (authors’ unpublished data, 2002). Moreover, unlike bradykinin, substance P cannot elicit the generation of an EET-like EDHF by porcine coronary arteries. The results show that although the endothelial cells investigated expressed a functional substance P receptor, this agonist failed to affect the intracellular transfer of Lucifer yellow. Taken together, these observations suggest that EETs, such as 11,12-EET, actively regulate gap junctional communication between endothelial cells. Actions of other endothelium-derived autacoids, such as NO and PGH$_2$, could be ruled out because almost identical responses were recorded in the absence and presence of L-NA and diclofenac.

EETs are potent intracellular signal transduction molecules that are included in the regulation of several kinase cascades (see review), but they have also been characterized as EDHFs and are reported to play a crucial role in the generation of NO/PGH$_2$-independent (EDHF-mediated) responses in the porcine coronary artery. To investigate the possible mechanisms underlying the EET-induced increase in cell coupling, we determined whether or not preventing the 11,12-EET-mediated hyperpolarization of endothelial cells could affect the initial increase in cell-cell coupling in either of the cell types used. The combination of charybdotoxin and apamin, $K_{Ca}$ channel inhibitors that abolish the EDHF-induced and 11,12-EET-induced hyperpolarization of isolated cells and intact vessels, did not influence either the transient increase or the sustained decrease in gap junctional communication. Moreover, depolarizing the endothelial cells with KCl (40 mmol/L) also failed to influence either phase of the agonist-induced change in electrical conductance or dye conductance coupling (authors’ unpublished data, 2001). Thus, although EETs activate large-conductance $K_{Ca}$ channels and although connexins are reported to be voltage sensitive, our results suggest that changes in the endothelial membrane potential, per se, do not affect dynamic EET-mediated changes in interendothelial coupling.

It has been suggested that ligand gating by intracellular messengers, such as cAMP, alters gap junctional open time, a phenomenon termed “mode shifting,” which might account for the dynamic regulation of gap junctional communication. In the present study, we observed that increases in cAMP markedly enhanced dye coupling between both types of endothelial cells studied. Moreover, in cells stimulated with cAMP released from a caged compound, enhanced coupling was paralleled by an alteration in the Triton X-100 solubility of Cx43, the predominant connexin isoform detected in the cells investigated. cAMP-induced alterations in connexin solubility have been reported in other cell types and are thought to reflect changes in connexin phosphorylation as well as the recruitment of intracellular connexins to the gap junction plaque.

Because cAMP has been proposed to act as a second messenger mediating cellular responses to 11,12-EET, we assessed the role of cAMP and PKA in agonist-induced changes in endothelial cell coupling. Not only did bradykinin and 11,12-EET enhance intracellular levels of cAMP, but preventing the activation of PKA abolished the agonist-induced and 11,12-EET–induced increase in endothelial cell coupling. Such observations suggest that gap junctional communication between endothelial cells can be activated by an agonist-stimulated, CYP-dependent, and cAMP-dependent pathway. Although the mechanism underlying the transient increase in interendothelial cell coupling remains to be assessed in intact vascular segments, some evidence exists to suggest that the agonist-induced facilitation of gap junctional communication is physiologically relevant. For example, in hamster retractor muscle arterioles, a more pronounced myoendothelial coupling was evident in acetylcholine-treated arteries than in arteries in which either smooth muscle or endothelial cells were stimulated by the injection of a negative current.

In the present study, we observed that the endothelial cell uncoupling observed 5 to 10 minutes after cell stimulation with either bradykinin or 11,12-EET was not detectable in cells pretreated with inhibitors of MEK, which prevent the activation of ERK1/2. Both bradykinin and 11,12-EET activated ERK1/2 in endothelial cells and induced a MEK inhibitor–sensitive shift in the mobility of Cx43 in SDS-PAGE, changes that are indicative of Cx43 phosphorylation. Such observations suggest that the activation of ERK1/2 leads to the phosphorylation of endothelial connexins and...
promotes cellular uncoupling. Indeed, both the electrical conductance and dye permeability of gap junctions have been shown to be sensitive to phosphorylation, and phosphorylation on serine and tyrosine residues has been linked to alterations in the conductance states of several connexins and/or decreases in gap junctional communication.\textsuperscript{32,33} Moreover, in HeLa cells, ERK1/2 is reported to phosphorylate three serine sites in the cytoplasmic carboxy-terminal domain of Cx43,\textsuperscript{21} a region of the protein that is thought to regulate the unitary conductance of gap junction channels.\textsuperscript{34}

Taken together, the results of the present investigation imply that an agonist-activated CYP 2C–dependent pathway exerts a dual effect on endothelial gap junctional communication that may play a crucial role in the phenomenon of ascending dilation and in the EDHF-mediated relaxation of small resistance-sized arteries. Although an increase in cAMP and the activation of PKA may underlie the agonist-induced facilitation of endothelial cell coupling, the subsequently observed decrease in cellular coupling appears to be attributable to the activation of ERK1/2, occurring partly as a direct consequence of the generation of 11,12-EET and partly by stimulation of the B\textsubscript{2} kinin receptor, per se.

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