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Cyclic AMP Mediates EDHF-Type Relaxations of Rabbit Jugular Vein

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Isolated rings of rabbit jugular vein have been used to test the hypothesis that formation of cAMP within the endothelial cell contributes to relaxations that are attributable to the endothelium-derived hyperpolarizing factor, EDHF. Relaxations induced by acetylcholine under conditions of combined NO synthase and cyclooxygenase blockade were almost abolished by inhibition of adenylate cyclase with the selective P-site agonist 2′,3′-dideoxyadenosine (2′,3′-DDA). They were similarly attenuated by the gap junction inhibitors 18α-glycyrrhetinic acid (18α-GA) and Gap 27 peptide which interrupt direct endothelium-smooth muscle communication without themselves affecting smooth muscle tone. By contrast, stimulation of adenylate cyclase with forskolin promoted gap junction-dependent relaxations, with concentration-relaxation curves to this agent exhibiting an equivalent rightward shift in the presence of 18α-GA and following endothelial denudation. The findings suggest that cAMP may cross from the endothelium to smooth muscle via gap junction channels and/or enhance the endothelial hyperpolarization normally associated with agonist stimulation. Both mechanisms may contribute to EDHF/gap junction-dependent relaxations.

Key Words: **endothelium-dependent hyperpolarizing factor; gap junctions; acetylcholine; 3′:5′-cyclic adenosine monophosphate.**

There is now evidence that the endothelium-derived hyperpolarizing factor (EDHF) that is widely thought to mediate NO- and prostanoid-independent agonist-induced vascular relaxation preferentially diffuses from the endothelium to smooth muscle via gap junctions, rather than the extracellular space (1–5). This hypothesis is supported by the existence of myoendothelial gap junction plaques in conduit arteries (6), and dye transfer experiments have also demonstrated direct chemical coupling between the two cell types (7). Gap junctions consist of 2 hemichannels contributed by apposing cells, with each being formed by 6 connexin protein subunits arranged around an aqueous central pore that provides electrical continuity and allows passage of small molecules <1 kDa in size (8). Connexin 43 (Cx43) protein is normally expressed in both endothelial and vascular smooth muscle cells (8–10), and in rabbit arteries EDHF-mediated relaxations are attenuated by a synthetic peptide homologous to the Gap 27 region of the second extracellular loop of this connexin subtype (1, 3, 4). EDHF-type relaxations are also inhibited by the 18α isoform of glycyrrhetinic acid which is a lipophilic aglycone that disrupts gap junction plaques (2). Gap 27 peptide and the 18β glycyrrhetinic acid isoform both attenuate the smooth muscle hyperpolarization associated with EDHF-type relaxations (3, 5).

EDHF-type responses are known to involve mobilization of arachidonic acid by a Ca^{2+}-dependent phospholipase A_2 (4, 11, 12), and arachidonic acid derivatives synthesized by the endothelium that have been proposed as candidate EDHFs include cytochrome P_{450} monooxygenase-derived epoxyeicosatrienoic acids (EETs) (13, 14) and N-arachidonylethanolamide (anandamide), an endogeneous cannabinoid (15) However, EDHF-, EET- and anandamide-induced relaxations and hyperpolarizations may display differential susceptibilities to K^+ channel blockers (16–20), and in some arteries EETs and anandamide may themselves stimulate endothelium-dependent relaxations that are sensitive to inhibition of gap junctional communication (4, 21). The identity of EDHF thus remains controversial. Indeed, it has been suggested that K^+ ions released from the endothelium may act as an EDHF by activating an inwardly-rectifying K^+ channel that promotes smooth muscle hyperpolarization (22). Recent evidence suggests, however, that this mechanism cannot be regarded as universal (23). Furthermore, on theoretical grounds it would seem unlikely that li-

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pophilic compounds such as EETs and anandamide diffuse from the endothelium via gap junctions as these channels are thought to allow the transfer of charged water-soluble species (8). Since there is compelling evidence for free intercellular transfer of cAMP and other nucleotides between cells (8, 24), and since elevations in intracellular cAMP are associated with vascular relaxation and hyperpolarization, in the present study we have evaluated the contribution of endothelial cAMP synthesis to the EDHF/gap junction-dependent phenomenon.

MATERIALS AND METHODS

Isolated ring preparations. Male New Zealand white rabbits (2-2.5 kg) were sacrificed by injection of sodium pentobarbitone (120 mg/kg; i.v.) and the jugular vein removed and transferred to Holman's buffer of the following composition (mM): 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, and 10 sucrose. The vessels were stripped of adherent connective and adipose tissue, and rings 2-3 mm wide cut and suspended in organ baths containing gassed (95% O_2, 5% CO_2, pH 7.4) buffer at 37°C. Tension was initially set between 0.2-0.3 g, and during an equilibrium period of 1 hr the tissues were repeatedly washed with fresh buffer and tension readjusted following stress relaxation. Endothelium-denuded rings were prepared by gentle abrasion of the intimal surface, and successful denudation subsequently confirmed by lack of response to 100 nM acetylcholine (ACh).

Experimental protocols. Endothelium-intact rings were preconstricted with 10 μM histamine and cumulative concentration-relaxation curves to ACh constructed. After washout, the vessels were preincubated for 40 mins with the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), 300 μM L-NAME + 10 μM indomethacin, or the guanylate cyclase inhibitor 1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one (ODQ, 10 μM). Further concentration-response curves to ACh were then constructed after reconstriction by histamine. Concentration-response curves to ACh were also obtained in the presence of L-NAME and indomethacin after 40 mins incubation with 100 μM 18α-GA, 300 μM Gap 27 peptide or the adenylate cyclase inhibitor 2′,3′-dideoxyadenosine (2′,3′-DDA, 30 μM). In a separate series of experiments, relaxations evoked by the adenylate cyclase activator forskolin were compared in endothelium-denuded rings and in endothelium-intact rings incubated with 300 μM L-NAME + 10 μM indomethacin in the presence and absence of 100 μM 18α-GA. ODQ was obtained from Affiniti, UK, and all other agents from Sigma, UK. Gap 27 peptide (amino acid sequence SRPTEKTIFII) was synthesised by Sigma Genosys, UK; purity was >95%.

Statistical analysis. All data are given as mean ± SEM, where n denotes the number of animals studied for each data point. Concentration-response curves were assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test. EC_{50} and maximal responses were compared by the Student's *t*-test for unpaired data. $P < 0.05$ was considered as significant.

RESULTS

Tension development by endothelium-intact rings of rabbit jugular vein in response to 10 μM histamine was 0.28 ± 0.02 g (n = 27). This was significantly increased (by ~15%) following preincubation with 300 μM L-NAME (n = 8), 300 μM L-NAME + 10 μM indomethacin (n = 14) or 10 μM ODQ (n = 5) ($P < 0.05$ in each case). Additional preincubation with 100 μM 18α-GA (n = 7), 300 μM Gap 27 peptide (n = 3) or 30 μM 2′,3′-DDA (n = 4) had no further effect on tone. The tension developed in endothelium-denuded preparations in response to 10 μM histamine was 0.33 ± 0.09 g (n = 4).

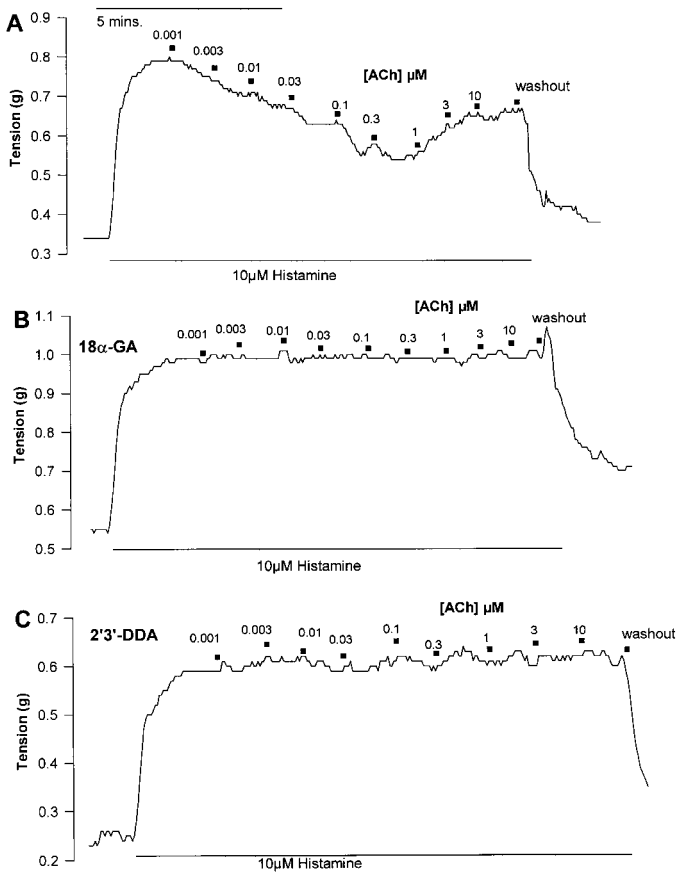

FIG. 1. (A) Representative trace showing concentration-dependent relaxations of rabbit jugular vein induced by acetylcholine (ACh) in the presence of 300 μM L-NAME and 10 μM indomethacin. Constriction at high concentrations of acetylcholine is attributable to direct effects on vascular smooth muscle. EDHF-type relaxations were abolished both by inhibition of gap junctional communication with 100 μM 18α-GA (B), or by inhibition of adenylate cyclase with 30 μM 2′, 3′-DDA (C).

In endothelium-intact rings ACh evoked concentration-dependent relaxations that reached a maximum of 96 ± 3% at a concentration of 300 nM (Figs. 1 and 2, n = 27). These relaxations were inhibited to a similar degree in the presence of either 300 μM L-NAME (to 29 ± 3%, n = 8, $P < 0.001$) or 10 μM ODQ (to 27 ± 3%, n = 5, $P < 0.001$). There was an equivalent rightward shift in EC_{50} values for ACh-induced relaxation in L-NAME (35 ± 11 nM, n = 8) and ODQ (49 ± 19 nM, n = 5) treated rings compared to endothelium-intact controls (16 ± 3 nM, n = 27, $P < 0.05$). Since ODQ attenuated ACh-induced relaxations to the same extent as L-NAME, direct cGMP-independent activation of K^+ channels by NO do not

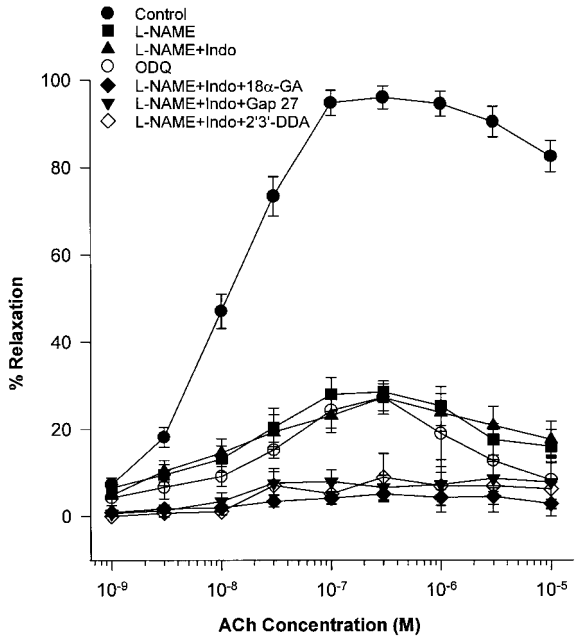

FIG. 2. Concentration-relaxation curves to acetylcholine (ACh) in endothelium-intact vessels. Control responses were attenuated to an equivalent extent by 300 μM L-NAME, the combination of 300 μM L-NAME and 10 μM indomethacin (Indo), and by 10 μM ODQ, an inhibitor of guanylate cyclase. EDHF-type relaxations observed in the presence of 300 μM L-NAME and 10 μM indomethacin were almost abolished by inhibition of gap junctions with 100 μM 18α-GA or 300 μM Gap 27 peptide and also by inhibition of adenylate cyclase with 30 μM 2′,3′-DDA.

contribute to EDHF-type relaxations in the rabbit jugular vein (25, 26). In endothelium-intact vessels treated with 300 μM L-NAME and 10 μM indomethacin, maximum relaxations to ACh were reduced to 27 $\pm$ 4% ($P <$ 0.001) in association with a shift in the EC_{50} value to 42 $\pm$ 11 nM (n = 14, $P < 0.05$). As inhibition was not enhanced by indomethacin, the contribution of endothelium-derived prostanoids to the EDHF-type relaxations can be excluded.

In the combined presence of 300 μM L-NAME + 10 μM indomethacin + 100 μM 18α-GA, relaxations were attenuated to 5.1 $\pm$ 1.8% (n = 7) at an ACh concentration of 300 nM which represented a significant reduction when compared to tissues treated with L-NAME and indomethacin alone ($P < 0.05$). 300 μM Gap 27 peptide and 30 μM 2′,3′-DDA similarly reduced maximum relaxations to ACh in L-NAME and indomethacin treated tissues to 8.7 $\pm$ 3.9% (n = 3, $P < 0.05$) and 9.0 $\pm$ 5.5% (n = 4, $P < 0.05$), respectively.

In endothelium-intact rings treated with 300 μM L-NAME + 10 μM indomethacin, forskolin-evoked relaxations attained a maximum of 114 $\pm$ 6% at 10 μM with an EC_{50} of 148 $\pm$ 48 nM (Fig. 3). In the additional presence of 100 μM 18α-GA, relaxations to forskolin were unaffected in terms of the maximal response (120 $\pm$ 7%), but there was a significant rightward shift in the EC_{50} value to 525 $\pm$ 121 nM (n = 5, $P < 0.05$). Endothelium-denuded preparations relaxed to forskolin with a maximal response of 116 $\pm$ 3% at 10 μM and an EC_{50} of 540 $\pm$ 205 nM (n = 4). These values were not significantly different from the those obtained in the presence of 18α-GA.

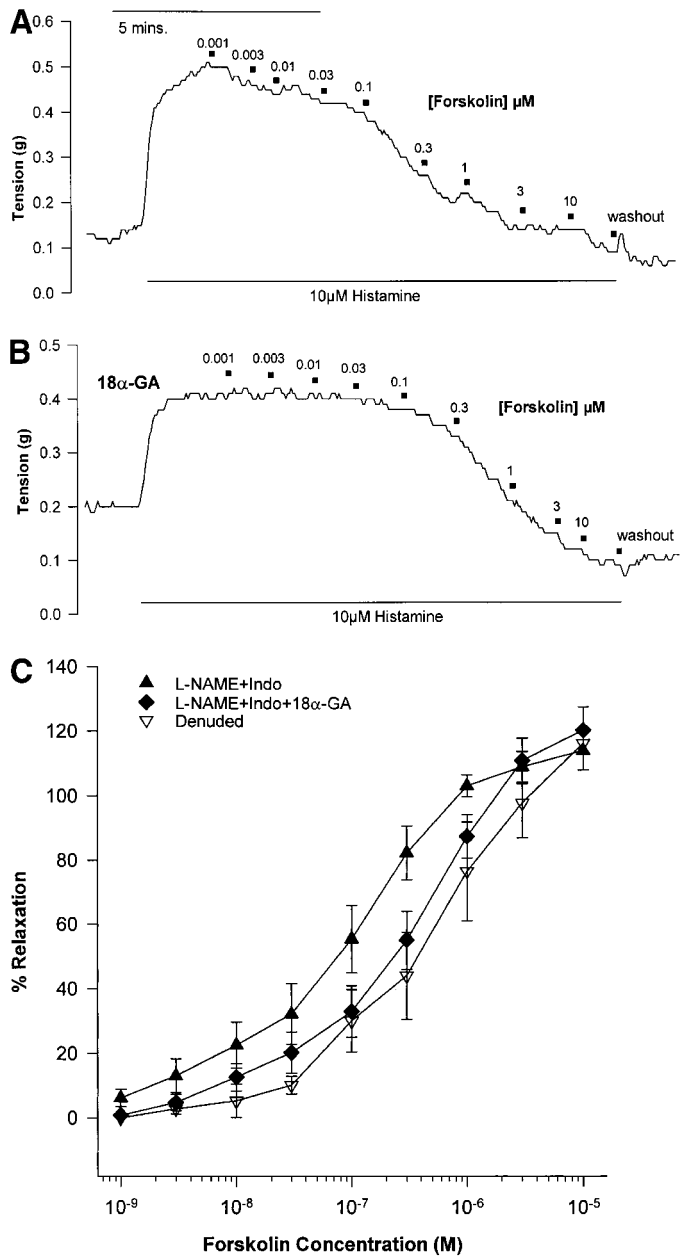

FIG. 3. (A, B) Representative traces showing that relaxations induced by forskolin in the presence of 300 μM L-NAME and 10 μM indomethacin were attenuated by 100 μM 18α-GA. Since forskolin stimulates adenylate cyclase, this suggests that cAMP stimulates gap junction-dependent relaxation. (C) Concentration-relaxation curves to forskolin in the presence and absence of 100 μM 18α-GA and in endothelium-denuded rings. Inhibition of gap junctional communication and loss of endothelium caused equivalent, statistically significant rightward shifts.

DISCUSSION

Gap junctional communication is essential for EDHF-type relaxations stimulated by agonists in a variety of rabbit arteries (1–4), and the present observations have confirmed the generality of this mechanism in veins from the same species. Experiments with the P-site agonist 2′,3′-DDA, and forskolin, which respectively inhibit and stimulate adenylate cyclase (27–29), have also provided new insights into the cellular mechanisms that underlie the EDHF phenomenon by demonstrating a central role for cAMP. Thus, 2′,3′-DDA and the two gap junction inhibitors 18α-GA and Gap 27 peptide almost abolished NO- and prostanoid-independent relaxations to ACh, whereas forskolin was significantly more potent as a vasodilator in endothelium-intact rings than in endothelium-denuded rings or intact rings incubated with 18α-GA. Taken together, these findings indicate that elevations in endothelial cAMP levels stimulate gap junction-dependent relaxation. Others have also reported NO-independent decreases in forskolin-induced relaxation following endothelial denudation in the rat mesenteric bed (30), and that the IC_{50} for cAMP accumulation in response to this agent is shifted ~3 fold to the right after removal of the endothelium in rabbit arteries (31). It has also been demonstrated that the endothelium is a major source of cAMP. In the perfused rat mesentery extracellular release of cAMP in response to ACh or the Ca^{2+}-ATPase inhibitor cyclopiazonic acid, which both evoke EDHF-type relaxations sensitive to Gap 27 peptide and 18α-GA (1–4), is markedly reduced by endothelial denudation (30, 32).

In many vessel types Ca^{2+}-activated K^+ channels (K_{Ca}) that mediate smooth muscle membrane hyperpolarization have been thought to be the principal target for EDHF (see 20 for review), and there is substantial evidence to suggest that elevations in cAMP increase the open state probability of K_{Ca} channels in vascular smooth muscle (27, 33–36). One possible explanation of the present findings, therefore, is that cAMP formed within the endothelium diffuses via gap junctions to reduce smooth muscle tone via protein kinase A (PKA)-mediated phosphorylation of such hyperpolarizing channels (34), phosphorylation of the myosin light chain kinase (37), and enhanced sequestration of Ca^{2+} within the sarcoplasmic reticulum (38). Preferential diffusion of cAMP via gap junctions would explain the well-known difficulties experienced in detecting downstream relaxations to EDHF in cascade bioassay (20) as this nucleotide is significantly less potent as a vasodilator when applied extracellularly than membrane permeant analogues such as 8-bromo-cAMP (39).

Cyclic AMP also exerts potent biological effects within the endothelial cell. Forskolin and membrane-permeable analogues of cAMP have thus been shown to activate K^+ channels that mediate endothelial hyperpolarization and enhance agonist-induced Ca^{2+} influx into the non-excitable endothelial cell (40). Since influx of extracellular Ca^{2+} is known to be essential for EDHF-type responses (41) and adenylate cyclase is a Ca^{2+}-dependent enzyme (29), elevations in endothelial cAMP could provide a mechanism whereby this hyperpolarization could become self-sustaining. Indeed, recent evidence suggests that K_{Ca} channels involved in EDHF-type relaxations may also be located on the endothelial cell (22, 42). Although endothelium-dependent agonists induce endothelial hyperpolarization directly (43, 44), it nevertheless remains controversial whether simple transmission of a hyperpolarizing current from the endothelium contributes to EDHF-type responses. In bovine ciliary artery, for example, endothelial hyperpolarization is conducted only as far as the immediately subjacent smooth muscle layer, perhaps reflecting the small mass of the endothelium relative to the media (45). However, the extent of smooth muscle hyperpolarization that results from electrotonic conduction would be expected to reflect the electrical input resistance of the media and it is possible that this differs between vessel types.

The gap junction-dependent pathways involved in EDHF-type relaxations distal to mobilization of arachidonic acid by phospholipase A_2 remain to be elucidated in detail, even though arachidonate metabolites such as EETs are known to activate K_{Ca} channels in both smooth muscle and endothelial cells (13, 14, 46). In rabbit mesenteric arteries the 5,6-EET regioisomer stimulates a strictly endothelium-dependent relaxation that is mediated in part via myoendothelial gap junctions and in part by NO (4). This gap junction dependent action of EETs could involve the generation of cAMP as in cardiac myocytes cAMP levels are elevated by exogeneous 11,12-EET and depressed by the cytochrome P_{450} inhibitor clotrimazole, leading to the hypothesis that EETs stimulate adenylate cyclase directly (47). 11,12-EET also mediates dilatation in renal arterioles through a mechanism that requires activation of PKA (48). The effects of EETs may nevertheless be species- and regio-specific as in bovine coronary artery 5,6-EET elevates cAMP levels, whereas other isomers do not (14).

Endothelial cells express Cxs 37, 40 and 43 (9), and vascular smooth muscle cells express Cx 43 (10) or Cxs 40 and 43 (49), thus in theory allowing endothelial-smooth muscle communication via homotypic, heterotypic and heteromeric gap junction channels. Early work suggested that PKA-mediated phosphorylation increases the conductance of Cx 43 gap junctions (50), although more recent studies suggest that the permeability of rat Cx 43 cardiac gap junctions is not altered by exposure to 8-bromo-cAMP (51, 52). Furthermore, this cAMP analogue reduces junction permeability by ~10% in human umbilical vein endothelial cells through an action which probably involves phosphorylation of Cxs 37 and 40 (53). Such evidence suggests

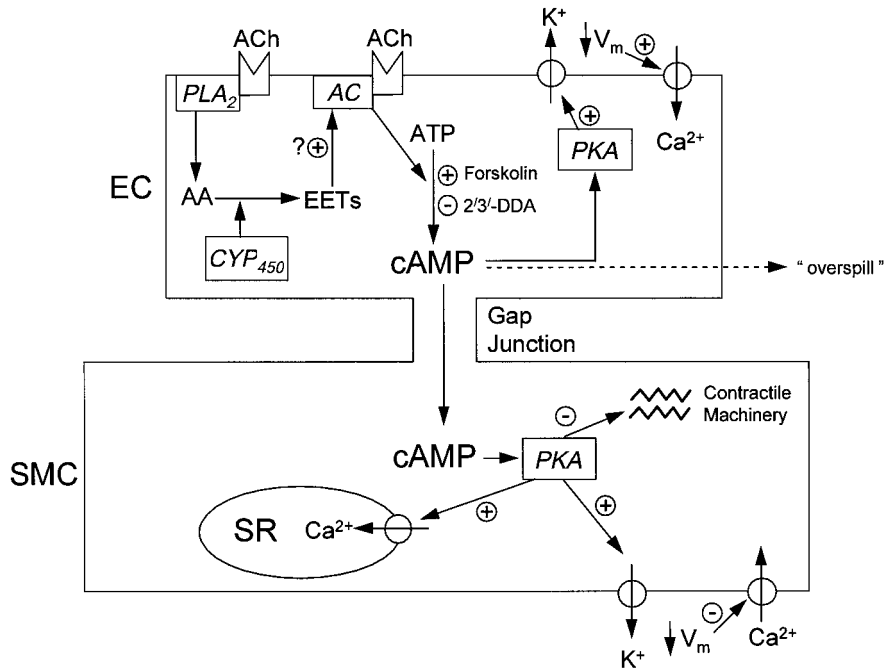

FIG. 4. Schematic showing the possible sites of action of cAMP in the mediation of EDHF-type relaxations. Efflux of K^+ ions results in membrane hyperpolarization, which promotes an increase in cytosolic free Ca^{2+} in the endothelial cell (EC), but a decrease in the smooth muscle cell (SMC). AC, adenylate cyclase; PKA, protein kinase A; PLA_2, phospholipase A_2; AA, arachidonic acid; CYP_{450}, cytochrome P_{450} monooxygenase; EETs, epoxyeicosatrienoic acids; V_m, membrane potential; SR, sarcoplasmic reticulum.

that cAMP does not increase the permeability of myoendothelial gap junctions and thereby promote the flux of signalling molecules into the media. In ovarian granulosa cells, however, PKA-dependent phosphorylation is thought to enhance direct intercellular communication (54). Further research is therefore necessary to determine the precise connexin composition of myoendothelial gap junctions and the role of cAMP in modulating their permeability.

In conclusion, we have provided evidence that the generation of cAMP within the endothelial cell underpins EDHF-type relaxations of the rabbit jugular vein. It is possible that cAMP not only crosses to smooth muscle cells via gap junctions but also enhances the endothelial cell hyperpolarization that is associated with the phenomenon (Fig. 4). The findings cannot, however, discount the possibility that cAMP-dependent phosphorylation events activate other pathways that converge to promote smooth muscle hyperpolarization and relaxation.

ACKNOWLEDGMENT

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