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REVIEW

Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis?

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An endothelium-derived hyperpolarizing factor (EDHF) that is distinct from nitric oxide (NO) and prostanoids has been widely hypothesized to hyperpolarize and relax vascular smooth muscle following stimulation of the endothelium by agonists. Candidates as diverse as K^+ ions, eicosanoids, hydrogen peroxide and C-type natriuretic peptide have been implicated as the putative mediator, but none has emerged as a 'universal EDHF'. An alternative explanation for the EDHF phenomenon is that direct intercellular communication *via* gap junctions allows passive spread of agonist-induced endothelial hyperpolarization through the vessel wall. In some arteries, eicosanoids and K^+ ions may themselves initiate a conducted endothelial hyperpolarization, thus suggesting that electrotonic signalling may represent a general mechanism through which the endothelium participates in the regulation of vascular tone.
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Abbreviations: ACh, acetylcholine; 2-APB, 2-aminoethoxydiphenyl borate; calcein-AM, calcein acetoxymethyl ester; cAMP, cyclic adenosine 3′,5′-monophosphate; CCE, capacitative Ca^{2+} entry; cGMP, cyclic guanosine 3′,5′-monophosphate; Cx, connexin; CYP_{450}, cytochrome P_{450} epoxygenase; 2′,5′-ddA, 2′,5′-dideoxyadenosine; 1-EBIO, 1-ethyl-2-benzimidazolinone; EET, epoxyeicosatrienoic acid; 14,15-EEZE, 14,15-epoxyeicosa-5(Z)-enoic acid; ER, endoplasmic reticulum; GA, glycyrrhetinic acid; IBMX, 3-isobutyl-1-methylxanthine; $InsP_3$, inositol 1,4,5-trisphosphate; K_{ATP}, ATP-sensitive K^+ channel; K_{Ca}, Ca^{2+}-activated K^+ channel; K_{ir}, inwardly-rectifying K^+ channel; K_v, voltage-dependent K^+ channel; LY320135, 6-methoxy-2-(4-methoxyphenyl) benzo[*b*]thien-3-yl)(4-cyanophenyl) methanone; NO, nitric oxide; ODYA, 17-octadecynoic acid; PLA_2, phospholipase A_2; Rp-cAMPS, Rp-8-bromoadenosine-3′,5′-cyclic monophosphorothioate; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase; Sp-5,6-DCI-cBIMPS, Sp-5,6-dichlorobenzimidazole-1-beta-D-ribofuranoside 3′,5′-cyclic phosphorothioate; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; TRAM-34, (1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole); TRAM-39, 2-(2-chlorophenyl)-2,2-diphenylacetonitrile; TRP, transient receptor potential; U73122, 1-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione

Introduction

Endothelial cells contribute to vascular control by releasing vasodilator prostanoids and NO when stimulated by agonists or fluid shear stress. Such stimuli also evoke an endothelium-dependent hyperpolarization of smooth muscle cells that is independent of NO and prostanoids, a phenomenon first described in guinea-pig, porcine and canine arteries exposed to acetylcholine (ACh) or bradykinin (Bolton *et al*., 1984; Bény & Brunet, 1988; Chen *et al*., 1988; Feletou & Vanhoutte, 1988). These observations led to the hypothesis that a distinct endothelium-derived hyperpolarizing factor, or EDHF, transfers across the extracellular space to modulate smooth muscle membrane potential (Chen *et al*., 1991; Mombouli *et al*., 1996; Popp *et al*., 1996; Gebremedhin *et al*., 1998). Despite over a decade of research, however, no single agent has emerged unambiguously as a 'universal EDHF' that mediates relaxation following release from the endothelium. Indeed, bioassay experiments with sandwich preparations constructed from closely apposed endothelium-intact and -denuded arterial strips, in which the donor endothelium and detector smooth muscle are electrically uncoupled, often fail to demonstrate relaxation in response to agonists such as ACh in the presence of an inhibitor of the constitutive endothelial NO synthase (eNOS) (Hecker *et al*., 1994; Plane *et al*., 1995; Chaytor *et al*., 1998; 2002; 2003; Hutcheson *et al*., 1999; Kagota *et al*., 1999). This raises the possibility that the EDHF phenomenon may involve passive electrotonic spread of hyperpolarization from the endothelium to smooth muscle cells *via* gap junctions, with relaxation being secondary to the closing of voltage-operated Ca^{2+} channels and associated reductions in the influx of extracellular Ca^{2+} ions that sustains contraction. Although experiments with agents that uncouple direct cell–cell communication nonspecifically, such as the long-chain alcohol heptanol and the anaesthetic halothane, initially failed to provide consistent evidence that gap junctions play a role in EDHF-type relaxations (Bény & Pacicca, 1994; Kühberger *et al*., 1994; Zygmunt & Hogestatt, 1996), there is growing evidence that more targeted inhibitors of direct cell–cell coupling do indeed attenuate endothelium-dependent smooth muscle hyperpolarization. A clear understanding of the

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participating mechanisms has nevertheless been clouded by the wide variety of signalling pathways that have been implicated in the EDHF phenomenon, raising questions as to which of the mechanisms involved might be central and which might be interactive consequences, and by the existence of species and vessel differences. This review will attempt to summarize the large matrix of data now available, and develop an integrative hypothesis that may reconcile some of the apparently conflicting observations reported in the literature.

Endothelial hyperpolarization: K^+ channel activation by intracellular Ca^{2+}

Agonists that evoke EDHF-type relaxations cause a rapid initial shift in the membrane potential of endothelial cells towards the reversal potential for K^+ (~ -80 mV), followed, variably, by stabilization some 10–20 mV below baseline or a slow return towards control that may sometimes be accompanied by an overshoot depolarization (Mehrke & Daut, 1990; Marchenko & Sage, 1993; Ohashi *et al*., 1999). The hyperpolarizing response is driven by the opening of Ca^{2+}-activated K^+ channels (K_{Ca}), as is evidenced by: (i) an associated efflux of Rb^+ ions from suitably loaded endothelial cells (Gordon & Martin, 1983), (ii) an inverse relationship between the amplitude of agonist-induced changes in endothelial membrane potential and the prevailing concentration of extracellular K^+ ions, such that hyperpolarization is converted to depolarization at $[K^+]_0 > 25$–50 mM (Mehrke & Daut, 1990) and (iii) attenuated hyperpolarization in the presence of peptide toxins such as apamin (a selective inhibitor of small-conductance channels, SK_{Ca}), charybdotoxin (an inhibitor of intermediate and large-conductance channels, IK_{Ca} and BK_{Ca}, and certain voltage-dependent K^+ channel subtypes, K_v) and iberiotoxin (a selective inhibitor of BK_{Ca} channels) (Edwards *et al*., 1998; Frieden *et al*., 1999; Ohashi *et al*., 1999). Activation of these different K_{Ca} subtypes may be agonist-specific and vary between native and cultured endothelial cells, in which K_{Ca} expression can alter during *passage*. Substance P and bradykinin, for example, hyperpolarize the native endothelium of porcine coronary arteries by opening SK_{Ca} and IK_{Ca} channels (Edwards *et al*., 2000; Bychkov *et al*., 2002). By contrast, cultured porcine coronary endothelial cells additionally express an iberiotoxin-sensitive BK_{Ca} channel that is activated by bradykinin, but not by substance P (Frieden *et al*., 1999). The participation of separate K_{Ca} subtypes explains observations that more than one toxin may be required to inhibit endothelial hyperpolarization completely. ACh-induced hyperpolarization of the endothelium of the rabbit aortic valve, for example, consists of a transient attenuated by charybdotoxin, but not apamin, and a sustained component that is partially attenuated by either toxin alone, with hyperpolarization being converted into depolarization by their combination (Ohashi *et al*., 1999).

The crucial physiological stimulus for the agonist-induced K_{Ca} channel activation that underpins endothelial hyperpolarization is an elevation in free cytosolic calcium, $[Ca^{2+}]_i$. Since the endothelium is devoid of voltage-gated Ca^{2+} channels and electrically nonexcitable, hyperpolarization will itself tend to promote elevations in $[Ca^{2+}]_i$ by enhancing the electrochemical gradient that drives transmembrane Ca^{2+} influx (Luckhoff & Busse, 1990; Kamouchi *et al*., 1999). However, it is now established that the principal mechanism that sustains the opening of endothelial K_{Ca} channels that follows agonist stimulation is capacitative Ca^{2+} entry (CCE) *via* membrane channels whose functionality is closely linked to depletion of the endoplasmic reticulum (ER) Ca^{2+} store (Marchenko & Sage, 1993; Sedova *et al*., 2000; Nilius & Droogmans, 2001). Agonist-evoked endothelial hyperpolarization may consequently be suppressed by lowering extracellular $[Ca^{2+}]$, by buffering elevations in $[Ca^{2+}]_i$ with a chelator, and by blocking CCE with 2-aminoethoxydiphenyl borate (2-APB), which reversibly inhibits store-operated Ca^{2+} channels (Chen & Suzuki, 1990; Marchenko & Sage, 1993; Frieden *et al*., 1999; Ohashi *et al*., 1999; Iwasaki *et al*., 2001; Bishara *et al*., 2002; Xie *et al*., 2002). In the converse sense, depletion of the ER by agents that prevent Ca^{2+} uptake by the SERCA pump, such as cyclopiazonic acid and thapsigargin, promote receptor-independent activation of endothelial K_{Ca} channels (Pasyk *et al*., 1995; Davis & Sharma, 1997; Fukao *et al*., 1997a). Agonist-induced depletion of the ER store is classically attributed to the activation of phospholipase C (PLC) *via* tyrosine phosphorylation, followed by the formation of inositol 1,4,5-trisphosphate ($InsP_3$), which releases Ca^{2+} from the store (Fleming *et al*., 1996). However, studies with triple $InsP_3$ receptor knockout B-lymphocytes suggest that additional pathways could also be involved, since CCE is not impaired in such cells (Ma *et al*., 2001). The nature of the store-operated Ca^{2+} influx mechanism thus remains controversial, but in endothelial cells may involve channels constructed from TRP proteins (Nilius *et al*., 2003). Whether the opening of store-operated channels is effected by direct mechanical coupling with the $InsP_3$ receptor or a diffusible cytosolic 'calcium influx factor' also remains the subject of debate (Kiselyov *et al*., 1998; Trepakova *et al*., 2000). Indeed, in endothelial cells there is evidence to support both hypotheses, since disruption of the cytoskeleton inhibits CCE, and store depletion may induce the formation of eicosanoid metabolites of arachidonic acid that promote an 2-APB-sensitive entry of Ca^{2+} ions *via* store-operated channels (Graier *et al*., 1995; Bishara *et al*., 2002; Xie *et al*., 2002; see below).

It has also become evident that the open-state probability of endothelial K_{Ca} channels can be modulated by secondary signalling events originating in the vascular media. Direct intercellular coupling *via* myoendothelial gap junctions may thus allow diffusion of $InsP_3$ and/or Ca^{2+} ions from activated smooth muscle cells into the endothelium, with the resulting elevation in $[Ca^{2+}]_i$ then promoting K_{Ca} channel-mediated endothelial hyperpolarization and depressing contraction through negative feedback (Dora *et al*., 1997; 2000; Yashiro & Duling, 2000; Budel *et al*., 2001). In theory, the ability of smooth muscle cells to modulate endothelial Ca^{2+} homeostasis could also contribute to the apparent paradox that K_{Ca} channel inhibitors reduce agonist-induced elevations in endothelial $[Ca^{2+}]_i$ in isolated cells (Kamouchi *et al*., 1999), but not in intact arteries (Yamanaka *et al*., 1998; Bolz *et al*., 1999; Ghisdal & Morel, 2001; Ungvari *et al*., 2002).

Relationship between endothelial hyperpolarization and smooth muscle relaxation

The electrical smooth muscle response that occurs during the EDHF phenomenon closely parallels that in the endothelium,

exhibiting an initial shift towards the reversal potential for K^+ followed by a slow return towards baseline (e.g. Figure 1). Evidence that activation of endothelial K_{Ca} channels by capacitative Ca^{2+} entry provides the driving force for relaxation is provided by observations that: (i) EDHF-type relaxations to ACh can be abolished by selective intimal application of K_{Ca} channel blockers, whereas adventitial application is without effect (Doughty *et al*., 1999), and (ii) that EDHF-type relaxations evoked by SERCA inhibitors such as cyclopiazonic acid are attenuated by K_{Ca} channel blockers (Dora *et al*., 2001). Formation of $InsP_3$, followed by depletion of the ER Ca^{2+} store, is likely to underpin the elevation in endothelial $[Ca^{2+}]_i$ that sustains agonist-induced smooth muscle hyperpolarization, since the PLC inhibitor U73122 attenuates subintimal hyperpolarizations evoked by ACh in the rat mesenteric artery (Fukao *et al*., 1997a), and attenuates EDHF-type relaxations/dilations in porcine coronary, rabbit mesenteric, guinea-pig carotid and rat middle cerebral arteries (Weintraub *et al*., 1995; Hutcheson *et al*., 1999; Quignard *et al*., 2002; You *et al*., 2002) and the perfused rat heart (Fulton *et al*., 1996). The ability of K_{Ca} channel inhibitors to attenuate EDHF-type responses without affecting agonist-induced increases in endothelial $[Ca^{2+}]_i$ in intact arterial preparations (see above) suggests that $[Ca^{2+}]_i$ does not serve as a primary activator of a putative Ca^{2+}-dependent 'EDHF synthase'. Indeed, in rat middle cerebral arteries, the directly acting IK_{Ca} agonist 1-EBIO, whose action is independent of PLC, evokes EDHF-type relaxations without elevating endothelial $[Ca^{2+}]_i$ above resting levels (Quignard *et al*., 2002; Marrelli *et al*., 2003).

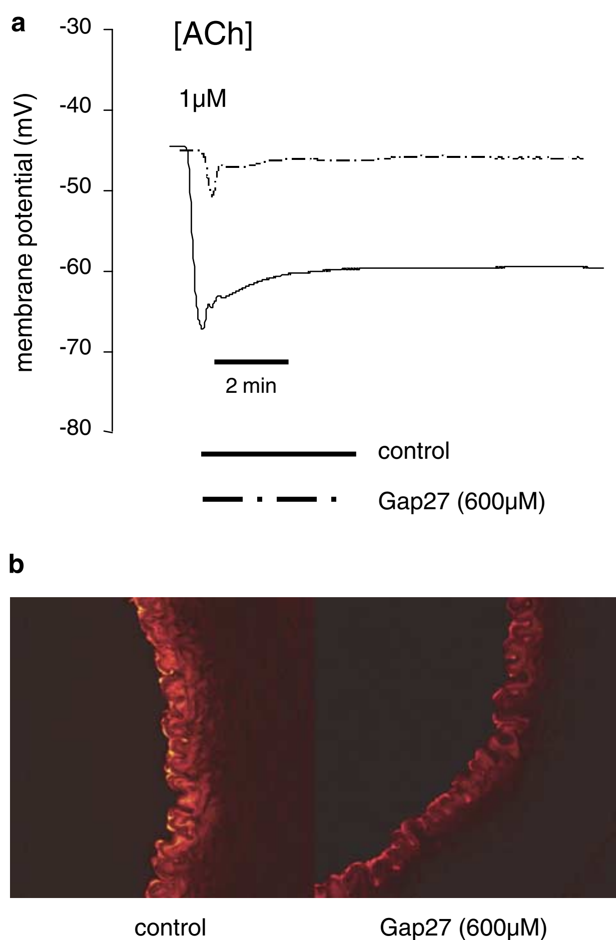


Figure 1 (a) Traces showing changes in subintimal smooth muscle cell potential in endothelium-intact strips of rabbit iliac artery impaled via the intima. ACh induced an initial shift towards the reversal potential for K^+, followed by a sustained hyperpolarization that was attenuated by [37,43]Gap27 (600 μM). (b) Confocal imaging of dye transfer from the endothelium into the media of rabbit femoral arteries following intraluminal perfusion and intimal loading with the cell-permeant tracer calcein AM. Subsequent cleavage of the acetoxymethyl moieties that permit endothelial uptake of this tracer results in a reduction in molecular mass from $\sim$1000 to $\sim$600 Da and allows diffusion of calcein (which is polar) through myoendothelial gap junctions. [37,43]Gap27 (600 μM) caused retention of calcein within the intima, thereby attenuating diffusion of the dye into subjacent smooth muscle cells (from Griffith *et al*., 2002).

Smooth muscle hyperpolarizations of the magnitude observed during the EDHF phenomenon are capable of causing marked reductions in vascular tone, because the voltage-dependent Ca^{2+} channels that maintain contraction are highly sensitive to the membrane potential (Nelson *et al*., 1990). Measurements during EDHF-type relaxations of pharmacologically constricted hamster skeletal muscle resistance arteries, rat renal arterioles and porcine coronary arteries thus reveal large and rapid falls in smooth muscle $[Ca^{2+}]_i$ to baseline levels, although, as with the associated electrical response, these may not always be sustained (Bolz *et al*., 1999; Marchetti *et al*., 2001; Ohnishi *et al*., 2001). While administration of apamin may sometimes be sufficient to abolish EDHF-type relaxations (Yamakawa *et al*., 1997; Ayajiki *et al*., 2000), in most vessels apamin and charybdotoxin are individually each only partially effective, and their co-administration necessary to attenuate relaxation completely (Edwards *et al*., 1998; Doughty *et al*., 1999). This observation parallels findings in isolated endothelial cells (see above), and has become widely regarded as a hallmark of the EDHF phenomenon. In many arteries, it is likely to reflect the dual participation of endothelial SK_{Ca} and IK_{Ca} channels, because the selective BK_{Ca} channel inhibitor iberiotoxin may be completely ineffective, even in combination with apamin, for example, in rat mesenteric, hepatic and renal arteries and guinea-pig coronary and basilar arteries (Rapacon *et al*., 1996; Petersson *et al*., 1997; Plane *et al*., 1997; Chataigneau *et al*., 1998a; Eckman *et al*., 1998; Edwards *et al*., 1998; Yamanaka *et al*., 1998). Furthermore, in rat carotid and mesenteric arteries, the sole involvement of SK_{Ca} and IK_{Ca} channels has been confirmed by using apamin in combination with the selective IK_{Ca} inhibitors TRAM-34 and TRAM-39 (Wulff *et al*., 2000; 2001; Eichler *et al*., 2003; Hinton & Langton, 2003). There may nevertheless be exceptions to the 'general' rule that SK_{Ca} and IK_{Ca} channels are the only K_{Ca} subtypes capable of participating in the EDHF phenomenon. BK_{Ca} channels can be detected immunohistochemically in the endothelium of rat skeletal muscle arterioles (Ungvari *et al*., 2002) and by patch-clamp techniques in the endothelium of the porcine aorta and renal artery (Papassotiriou *et al*., 2000; Brakemeier *et al*., 2003), and the ability of iberiotoxin to inhibit EDHF-type relaxations in rabbit renal and femoral arteries could reflect the expression of BK_{Ca} channels, which has been documented electrophysiologically in freshly isolated endothelial cells from this species (Rusko *et al*., 1992; Kagota *et al*., 1999; Kwon *et al*., 1999; Yousif *et al*., 2002).

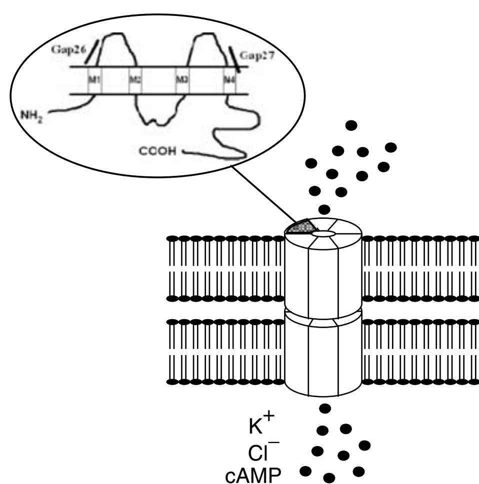
Despite the close relationship between endothelial and smooth muscle hyperpolarization, the nature of the pathways

that allow changes in endothelial membrane potential to be translated into smooth muscle hyperpolarization, reductions in smooth muscle $[Ca^{2+}]_i$ and relaxation remain controversial. As outlined above, there are two principal hypotheses, namely, electrotonic signalling *via* gap junctions and diffusion of freely transferable mediators across the extracellular space.

Gap junctional communication

Gap junctions are formed by the docking of two hemichannels (called connexons) at points of cell–cell contact, with each hemichannel being constructed from six connexin (Cx) protein subunits that traverse the cell membrane four times to expose two extracellular peptide loops (Kumar & Gilula, 1996; Perkins *et al*., 1998). Interdigitation of the extracellular loops of apposing connexons, followed by a 30° rotation, creates an aqueous central pore that allows intercellular diffusion of ions and signalling molecules <1 kDa in size, thereby conferring electrical continuity between coupled cells (see the schematic representation in Figure 2). Individual gap junctions may subsequently aggregate in plaques that consist of focal clusters of many hundreds of units in the cell membrane and whose characteristic pentalaminar appearance can be visualized by electron microscopy at points of hetero- and homocellular contact between endothelial and smooth muscle cells (Spagnoli *et al*., 1982; Sandow & Hill, 2000). The formation of such structures is thought to facilitate cooperative interactions between their component gap junctions and thereby strongly enhance intercellular signalling (Bukauskas *et al*., 2000). Indeed, in communication-incompetent Neuro-2a, HeLa and RIN cells, stably transfected to express a fluorescent connexin protein, the extent of dye transfer and electrical communication between cell pairs correlates closely with plaque size, and coupling is absent in the absence of detectable plaques, even when connexin protein is diffusely present in the cell membrane (Bukauskas *et al*., 2000). Three main connexin subtypes, classified as Cxs 37, 40 and 43 according to molecular mass in kDa, are widely distributed in the vasculature, and some vessels may also express Cx45. Immunostaining of the vessel wall with specific antibodies reveals the distinctive punctate appearance of plaques containing these connexins, with protein expression generally being more abundant in the endothelium than the media, and different connexin subtypes often co-localizing in the same plaque (Haas & Duling, 1997; Hong & Hill, 1998; Yeh *et al*., 1998; Chaytor *et al*., 2001; Li & Simard, 2001; Berman *et al*., 2002; Rummery *et al*., 2002; Ujiie *et al*., 2003). In addition to homotypic gap junctions, in which each connexon contains the same connexin subtype, heterotypic channels, in which each connexon is constructed from a different connexin subtype, and heteromeric channels, in which each connexon contains mixtures of subtypes, may therefore be present in the vascular wall. The existence of such hybrid gap junctions has been confirmed electrophysiologically in cultured smooth muscle cells and isolated arteries by patch-clamp identification of channels with complex conductances consistent with the formation from more than one connexin protein (He *et al*., 1999; Li & Simard, 1999; 2001; Wang *et al*., 2001; Yamazaki & Kitamura, 2003).

Attempts to demonstrate direct communication between endothelial and smooth muscle cells by microinjection of fluorescent tracer dyes into individual endothelial cells are often unsuccessful (Segal & Bény, 1992; Little *et al*., 1995; Jiang *et al*., 2001; Yamamoto *et al*., 2001). This observation may, at least in part, reflect the preferential diffusion of dye within the endothelial monolayer *via* large inter-endothelial plaques, rather than radial diffusion into the media *via* myoendothelial gap junction plaques which are much smaller and less numerous (Haas & Duling, 1997; Sandow & Hill, 2000). Loading the entire endothelial layer with a lipophilic tracer such as calcein AM, which is cleaved intracellularly after uptake, can overcome this 'sink–source' problem by allowing diffusion of the polar fluorescent product calcein from the endothelium into the media (Figure 1). It should be appreciated, however, that failure to demonstrate dye coupling between adjacent cells does not necessarily imply the absence



	Gap 26 domain	Gap 27 domain
Cx37	VCYDQAFPISHIR	SRPTEKTIFII
Cx40	VCYDQAFPISHIR	SRPTEKNVFIV
Cx43	VCYDKSFPISHVR	SRPTEKTIFII

Figure 2 Schematic representation showing the structure of a connexin protein and how six such elements form a connexon. Docking of connexons from apposing cells results in the formation of an aqueous pore that allows the transfer of ions and small signalling molecules between coupled cells. Each connexin possesses four transmembrane segments (M1–4). Also highlighted are the Gap 26 and Gap 27 domains of the first and second extracellular connexin loops which are conserved in man, rat and mouse. Differences in the amino-acid sequences of the Gap 26 and Gap 27 domains of the three major vascular connexins allow designation as ^{37,40}Gap26, ^{43}Gap26, ^{37,43}Gap27 and ^{40}Gap27. Synthetic peptides possessing these sequences inhibit direct intercellular communication in a connexin-specific fashion.

of electrical continuity, since the efficacy of dye transfer is influenced by the molecular mass and charge of the specific tracer employed, as well as the nature of the connexin subtypes expressed in the cells under study (Little *et al*., 1995; Kruger *et al*., 2002). Indeed, in hamster cheek pouch arterioles, hamster skeletal muscle feed arteries and guinea-pig mesenteric arterioles, electrophysiological studies have confirmed that endothelial hyperpolarizations evoked by ACh or direct current injection can be detected synchronously in smooth muscle cells, and conversely, that action potentials originating in smooth muscle cells are conducted to the endothelium (Segal & Bény, 1992; Emerson & Segal, 2000; Coleman *et al*., 2001a; Yamamoto *et al*., 2001). In porcine coronary artery and rat aorta oscillations in smooth muscle membrane potential also drive synchronous electrical fluctuations in the endothelium, generalizing such observations to conduit vessels (von der Weid & Bény, 1993; Marchenko & Sage, 1994). In guinea-pig mesenteric arterioles, gap junctions coupling endothelial and smooth muscle cells behave as simple ohmic resistors without rectification, since electrical responses transmitted in either direction are reduced by 10–20% in amplitude without alteration in their dynamic temporal form (Yamamoto *et al*., 2001). In rat pial arterioles, however, dual-electrode macroscopic current recordings from adjacent endothelial and smooth muscle cells demonstrate partial rectification, as evidenced by an asymmetric transjunctional conductance–voltage relationship that is likely to reflect the presence of heterotypic and/or heteromeric myoendothelial gap junctions (Yamazaki & Kitamura, 2003).

Despite compelling experimental evidence for direct electrical coupling between the endothelium and the media, it has nevertheless been argued that nonregenerative spread of endothelial hyperpolarization into subjacent smooth muscle cells will fail to contribute to relaxation in conduit arteries, on the basis that the large relative mass of the media will dissipate passive electrical signals originating from the endothelium by acting as a current sink (Bény, 1999). Evidence to support the contrary view is summarized in the following sections.

Connexin-mimetic peptides

Minor variations in the amino-acid sequences of the first and second extracellular loops of Cxs 37, 40 and 43 permit the synthesis of short synthetic peptides that are homologous to the conserved Gap 26 and 27 domains of these proteins and are capable of interrupting gap junctional communication following relatively short incubations (15–40 min) at concentrations in the range 300 μM–1 mM (Chaytor *et al*., 1997; 1998; 1999; 2001; Dora *et al*., 1999; Berman *et al*., 2002; Griffith *et al*., 2002; Figure 2). Experiments with cultured cells suggest that the inhibitory effects of such peptides against cell–cell coupling are connexin specific. In confluent COS fibroblasts, for example, in which the only connexin protein expressed is Cx43, intercellular transfer of the dye Lucifer yellow is attenuated by ^{37,43}Gap 27, a peptide possessing homology with the Gap 27 domain of the second extracellular loop of Cx43 (and also Cx37), but not by ^{40}Gap 27, which is homologous to the corresponding domain of Cx40 and differs by just three amino acids (Chaytor *et al*., 1999). Since similar concentrations of ^{37,43}Gap 27 and ^{43}Gap 26 are equally effective in attenuating dye transfer between HeLa cells transfected to express Cx43, it is apparent that first and second loop peptides may be used interchangeably to inhibit gap junctional communication (Berman *et al*., 2002).

Evidence that the role of direct endothelial–smooth muscle coupling is central to the EDHF phenomenon has been provided by observations that connexin-mimetic peptides attenuate subintimal smooth muscle hyperpolarizations and associated relaxations evoked by ACh, ATP, substance P and bradykinin in large arteries and veins from the rabbit, rat, pig and guinea-pig (Chaytor *et al*., 1998; 1999; 2001; 2003; Dora *et al*., 1999; Edwards *et al*., 1999; 2000; Griffith & Taylor, 1999; Hutcheson *et al*., 1999; Doughty *et al*., 2000; Berman *et al*., 2002; Griffith *et al*., 2002; Sandow *et al*., 2002; Xu *et al*., 2002; Ujiie *et al*., 2003). Although their molecular mechanism of action remains to be established, ^{43}Gap 26 and ^{37,43}Gap 27 do not perturb the physical stability of plaques constructed from Cx43, despite attenuating dye transfer (Berman *et al*., 2002). This suggests that connexin-mimetic peptides modulate channel gating rather than connexon docking, and is consistent with an ability to attenuate EDHF-type relaxations in a reversible fashion, with coupling being restored on peptide washout (Chaytor *et al*., 1998; 2001). An action downstream of specific membrane receptors has been confirmed by observations that ^{37,43}Gap 27 attenuates receptor-independent EDHF-type relaxations evoked by the SERCA inhibitor cyclopiazonic acid, and does not impair ACh-induced endothelial NO synthesis in sandwich bioassay experiments (Chaytor *et al*., 1998). Importantly, connexin-mimetic peptides do not suppress endothelial hyperpolarization directly, and they do not influence mechanical smooth muscle responses to exogeneous nitrovasodilators, K_{ATP} channel openers or constrictor agonists (Chaytor *et al*., 1997; 1998; 2001; Dora *et al*., 1999; Richards *et al*., 2001; Sandow *et al*., 2002; Ujiie *et al*., 2003). In addition to attenuating electrotonic signalling *via* myoendothelial gap junctions, ^{37,43}Gap 27 impedes the transfer of fluorescent tracer dye from the endothelium into the media of rabbit ilio-femoral arteries (Figure 2), thus providing evidence that connexin-mimetic peptides are capable of interrupting direct chemical signalling *via* myoendothelial gap junctions (Griffith *et al*., 2002).

Connexin-mimetic peptides also uncouple vascular smooth muscle cells (Chaytor *et al*., 1997), so that in some arteries their inhibitory effects against the EDHF phenomenon may reflect an ability to attenuate electrotonic signalling within the media. In porcine coronary arteries stimulated by substance P, for example, comparative measurements of subintimal and adventitial smooth muscle membrane potential suggest that ^{37,43}Gap 27 preferentially depresses the relay of agonist-induced hyperpolarizing signals originating in the endothelium through successive layers of smooth muscle cells (Edwards *et al*., 2000). Whole-cell patch clamping of smooth muscle cells in the media of rat cerebral pial arterioles has also demonstrated that ^{37,43}Gap 27 markedly impairs current flow to neighbouring cells, and thereby increases smooth muscle membrane input resistance (Yamazaki & Kitamura, 2003). Whether direct electrical coupling within the endothelium contributes to the EDHF phenomenon remains to be established. However, it may be speculated that the presence of large inter-endothelial gap junction plaques will allow this monolayer to function as a low-resistance current source, thereby facilitating transmission of hyperpolarization into the

media *via* high-resistance myoendothelial gap junctions (Haas & Duling, 1997; Sandow & Hill, 2000; Chaytor *et al*., 2001; Yamamoto *et al*., 2001). In support of this concept, pharmacological uncoupling of gap junctions increases the input electrical resistance of the endothelium of guinea-pig mesenteric arterioles by $\sim$150-fold (Yamamoto *et al*., 1998).

Although connexin-mimetic peptides targeted against a single connexin subtype are capable of causing substantial reductions in EDHF-type responses in specific arteries, there is emerging evidence that peptide combinations directed against more than one subtype are generally more effective (Table 1). This is likely to reflect variations in the size, frequency, location and connexin composition of the gap junction plaques present in different vessels, and an ability of channels constructed from different connexin proteins to compensate for each other functionally. In confluent rat aortic smooth muscle A7r5 cells, which are highly coupled by numerous plaques constructed from Cxs 40 and 43, dye transfer is unaffected by ^{40}Gap 27 and ^{43}Gap 26 administered individually at concentrations of 600 μM, but is inhibited by dual administration of the peptides at the same net overall concentration, that is, 300 μM each (Chaytor *et al*., 2001). Analogously, in the rabbit iliac artery, in which immunostaining reveals plaques containing Cxs 37 and 40 in the endothelium and Cxs 40 and 43 in the media, the triple peptide combination ^{37,43}Gap 27 + ^{43}Gap 26 + ^{40}Gap 27 (at 300 μM each) effectively abolishes subintimal EDHF-type smooth muscle hyperpolarizations evoked by the Ca^{2+} ionophore A23187, whereas ^{37,43}Gap 27 alone attenuates by $\sim$65% at an equivalent total peptide concentration of 900 μM (Chaytor *et al*., 2003). These observations suggest that more than one connexin subtype contributes to myoendothelial coupling in this vessel. The inhibitory effects of single peptides and peptide combinations against ACh-evoked relaxations in rabbit ear, rabbit middle cerebral and rat hepatic arteries are also complex, and may additionally reflect heterogeneity in the patterns of connexin protein expressed in the media. In these vessels, endothelial gap junction plaques contain Cxs 37, 40 and 43, albeit in slightly different ratios, whereas the connexin composition of smooth muscle plaques is more vessel specific. In rabbit ear arteries, Cx43 is the sole subtype detectable in medial plaques on immunostaining and ^{37,43}Gap 27 and ^{43}Gap 26 both abolish EDHF-type relaxations, consistent with a dominant role for gap junctions containing Cx43 (Berman *et al*., 2002). In rabbit middle cerebral arteries, Cxs 40 and 43 are both present in the media, and ^{37,43}Gap 27 and ^{40}Gap 27 partially attenuate relaxation, with responses being almost eliminated by the two peptides in combination (Ujiie *et al*., 2003). In the rat hepatic artery, Cxs 37 and 43 are both highly expressed in medial plaques and EDHF-type relaxations are unaffected by individual peptides, whereas a combination simultaneously targeting Cxs 40 and 43 (^{43}Gap 26 + ^{40}Gap 27) attenuates by $\sim$50% and the triple combination ^{37,43}Gap 27 + ^{43}Gap 26 + ^{40}Gap 27 inhibits relaxation almost completely (Chaytor *et al*., 2001). The participation of different connexin subtypes in the EDHF phenomenon is also evident *in vivo*, as intrarenal infusion of ^{40}Gap 27 and ^{37,43}Gap 27 each attenuate the increase in renal blood flow induced by ACh in anaesthetized rats (De Vriese *et al*., 2002).

Heterogeneous connexin expression could also explain more major differences in electrotonic signalling. In the rat femoral artery, for example, some workers have documented large

Table 1 Semiquantitative analysis of connexin expression and the effects of connexin-mimetic peptides on Lucifer yellow dye in rat smooth muscle cells and EDHF-type hyperpolarizations and relaxations in rabbit and rat arteries

Connexins identified/peptide inhibitors		*Cx37*	*Cx40*	*Cx43*	*^{37,43}Gap 27*	*^{43}Gap 26*	*^{40}Gap 27*	*^{37,43}Gap27 + ^{40}Gap 27*	*^{43}Gap 26 + ^{40}Gap 27*	*^{37,43}Gap 26 + ^{43}Gap 26 + ^{40}Gap 27*
A7r5 rat aortic myocytes dye transfer		–	++	++		–(600 μM)	–(600 μM)		**(600 μM)	
Rabbit iliac artery A23187 hyperpolarization	E	+	+	–	*(900 μM)			**(900 μM)		***(900 μM)
	SM	–	+	+						
Rabbit ear artery ACh relaxation	E	+	+	+	***(300 μM)	***(300 μM)				
	SM	–	–	+						
Rabbit middle cerebral artery ACh relaxation	E	+	+	+	*(300 μM)		*(300 μM)	***(600 μM)		
	SM	–	+	+	**(600 μM)		**(600 μM)			
Rat hepatic artery ACh relaxation	E	+	+	+	–(600 μM)	–(600 μM)	–(600 μM)	*(600 μM)	*(600 μM)	***(900 μM)
	SM	++	–	++						

+/– denote the presence/absence of Cxs 37, 40 and 43 in endothelial (E) and smooth muscle (SM) gap junction plaques as visualized by antibody staining. Functional inhibition by connexin-mimetic peptides (denoted as */–) was estimated at the total peptide concentrations indicated in brackets. The data provide evidence that more than one connexin subtype may contribute to electrotonic signalling in the vessel wall and that the efficacy of connexin-mimetic peptides as inhibitors of the EDHF phenomenon in different vessels is likely to reflect heterogeneity in the size, frequency, location and connexin composition of gap junction plaques. Data compiled from Chaytor *et al*. (2001, 2003); Berman *et al*. (2002); Ujiie *et al*. (2003).

EDHF-type responses to ACh (Savage *et al*., 2003), whereas others have reported an almost complete absence of relaxation (Zygmunt *et al*., 1995; Sandow *et al*., 2002). Notably, the absence of a conducted subintimal smooth muscle response following hyperpolarization of the endothelium by ACh has been correlated with a lack of myoendothelial gap junctions, as assessed by serial electron microscopy in this vessel (Sandow *et al*., 2002). Such variability could conceivably reflect differences in strain or age, since the frequency of endothelial–smooth muscle contacts made *via* fenestrations in the internal elastic lamina decreases during maturation (Kristek & Gerova, 1997). Major regional variations are also evident in porcine vessels: in the ciliary artery, agonist-induced endothelial hyperpolarization is only occasionally transmitted to immediately subjacent smooth muscle cells, and is consequently undetectable deep in the media, in marked contrast to the porcine coronary artery (Pacicca *et al*., 1996; Bény *et al*., 1997; Edwards *et al*., 2000). Differences in connexin expression could also influence the space constant for propagation of hyperpolarization through the media (i.e., the distance over which a change in smooth muscle membrane potential decrements by a factor of $1/e$ to $\sim$37% of its original value). This parameter can be estimated to be of the order of 1–2 mm in porcine coronary and rabbit iliac arteries, but just 50–100 μm in guinea-pig mesenteric arterioles denuded of their endothelium (Pacicca *et al*., 1996; Yamamoto *et al*., 2001; Griffith *et al*., 2002).

Glycyrrhetinic acid derivatives

Gap junctional communication can also be inhibited by the 18α- and 18β-isoforms of glycyrrhetinic acid (GA), a lipophilic steroidal aglycone derived from glycyrrhizic acid that is found in the liquorice root *glycyrrhizia glabra*, and by carbenoxolone, a water-soluble hemisuccinate derivative of 18β-GA (Davidson *et al*., 1986; Davidson & Baumgarten, 1988). EDHF-type relaxations of rabbit superior mesenteric arteries and jugular veins evoked by ACh are attenuated by these compounds, with rank inhibitory potencies being 18β-GA > 18α-GA $\gg$ carbenoxolone in the superior mesenteric artery, suggesting that the decreased lipophilicity of carbenoxolone reduces its ability to inhibit gap junctional communication in the vessel wall (Taylor *et al*., 1998; Griffith & Taylor, 1999; Chaytor *et al*., 2000). Consistent with an action distal to the occupation of specific membrane receptors, 18α-GA also attenuates EDHF-type relaxations evoked by the SERCA inhibitor cyclopiazonic acid (Taylor *et al*., 1998). Evidence from a spectrum of rat and guinea-pig arteries suggests that GA derivatives, like connexin-mimetic peptides, inhibit EDHF-type relaxations by impairing the electrotonic spread of endothelial hyperpolarization into and through the vascular media *via* gap junctions (Edwards *et al*., 1999; Yamamoto *et al*., 1999; Jiang *et al*., 2001; Yamazaki & Kitamura, 2003). Of the three commonly employed derivatives, 18α-GA has emerged as the most suitable pharmacological probe for assessing the contribution of gap junctions to endothelium-dependent relaxation. In rabbit arteries, 18α-GA inhibits EDHF-type responses at concentrations that do not affect smooth muscle tone, whereas 18β-GA and carbenoxolone both relax smooth muscle directly, and carbenoxolone functionally enhances NO activity (Dembinska-Kiec *et al*., 1991; Chaytor *et al*., 2000). There is also evidence that 18β-GA and carbenoxolone nonspecifically depress endothelial hyperpolarization (Tare *et al*., 2002), although this has not been a universal finding, and does not appear to be a problem with 18α-GA (Edwards *et al*., 1999; Murai *et al*., 1999; Yamamoto *et al*., 1999; Jiang *et al*., 2001). Nonspecific 'toxic' effects of GA derivatives are also more pronounced with the β than the α configuration in nonvascular cells (Davidson *et al*., 1986; Davidson & Baumgarten, 1988; Goldberg *et al*., 1996).

In contrast to the reversible action of connexin-mimetic peptides, inhibition of EDHF-mediated relaxations of rabbit arteries by 18α-GA becomes irreversible following 1 h incubation (Chaytor *et al*., 1998; 2000), an observation that may reflect the ability of GA derivatives to disrupt gap junction plaques. In liver and alveolar epithelial cells expressing Cx43, GA derivatives cause time- and concentration-dependent dephosphorylation of this connexin subtype, plaque disassembly and internalization, with progressive reductions in the expression of Cx43 becoming evident as exposure times are extended beyond 30 min (Guan *et al*., 1996; Guo *et al*., 1999). By contrast, the initial interruption of intercellular communication is rapid (within 15–30 min), reversible, and not associated with changes in the integrity of gap junction plaques or the phosphorylation status of connexin proteins (Guan *et al*., 1996; Guo *et al*., 1999). The time course of these observations correlates closely with the ability of 18α-GA to decrease the macroscopic junctional current that flows between pairs of coupled rat pial arteriolar smooth muscle cells following depolarizing voltage steps and the number of unitary channel events that can be detected as the cells become progressively uncoupled (Yamazaki & Kitamura, 2003). The molecular mechanisms that underlie the action of GA derivatives remain unknown, and a definitive link between connexin dephosphorylation and the stability of gap junction plaques remains to be established. Indeed, in C6 glioma cells, the water-soluble hemisuccinate form of 18α-GA causes plaque disaggregation without affecting the phosphorylation status of Cx43 (Goldberg *et al*., 1996), and 18α-GA itself attenuates dye transfer between HeLa cells transfected to express functional gap junctions constructed from Cx26, a connexin subtype that is not regulated by phosphorylation (George *et al*., 1998). Another lipophilic compound, palmitoleic acid, is also known to interrupt gap junctional communication and inhibit EDHF-type hyperpolarizations and relaxations, although again its mechanism of action remains to be delineated (Harris *et al*., 2000; Kenny *et al*., 2002; Ungvari *et al*., 2002).

Permissive role of cyclic AMP

In many nonvascular cell types, elevations in cAMP levels enhance cell–cell coupling *via* gap junctions constructed from Cxs 40 and 43 through poorly understood mechanisms that involve connexin phosphorylation by protein kinase A (PKA) and/or rapid recruitment of connexin protein to the cell membrane (Burghardt *et al*., 1995; Chanson *et al*., 1996; Abudara *et al*., 2000; Paulson *et al*., 2000; van Rijen *et al*., 2000; Gladwell & Jefferys, 2001; Grazul-Bilska *et al*., 2001). The ability of this cyclic nucleotide to modulate intercellular communication is also evident in the vascular wall as 8-bromo-cAMP (a cell-permeant analogue) and the cAMP phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) enhance

the diffusion of fluorescent tracer dye from the endothelium into the media *via* gap junctions in rabbit ilio-femoral arteries (Griffith *et al*., 2002). Endogenous formation of cAMP may therefore play an important role in the EDHF phenomenon, since agonists such as ACh are capable of promoting endothelial synthesis of the nucleotide through a mechanism that is independent of the formation of prostanoids (Kamata *et al*., 1996; Taylor *et al*., 2001). Indeed, in rabbit ilio-femoral arteries, the adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine (2′,5′-ddA) markedly attenuates subintimal smooth muscle hyperpolarizations evoked by ACh, thus suggesting that cAMP generated within the endothelium plays an important role in facilitating electrical coupling *via* myoendothelial gap junctions during agonist-induced responses (Griffith *et al*., 2002). This permissive role of cAMP is likely to explain the ability of 2′,5′-ddA and the PKA inhibitor Rp-cAMPS to attenuate EDHF-type relaxations in rabbit arteries (Taylor *et al*., 2001; Chaytor *et al*., 2002; Griffith *et al*., 2002).

The pathways that underpin prostanoid-independent endothelial cAMP synthesis nevertheless remain unclear. In theory, they could involve stimulation of specific Ca^{2+}-stimulated adenylyl cyclase isoforms by elevations in $[Ca^{2+}]_i$, since there is evidence that in nonexcitable cells such isoforms co-localize with store-operated Ca^{2+} channels in the plasma membrane, where they are preferentially regulated by CCE (Burnay *et al*., 1998; Watson *et al*., 1998; Smith *et al*., 2002). This scenario would explain the ability of the SERCA inhibitor cyclopiazonic acid to promote cAMP efflux from rat endothelial cells (Kamata *et al*., 1996) and to evoke EDHF-type relaxations of rabbit arteries that, like those induced by ACh, are attenuated by inhibition of adenylyl cyclase by 2′,5′-ddA (Taylor *et al*., 1998; 2001). However, there is also evidence that gap junction-dependent elevations in endothelial Ca^{2+} secondary to smooth muscle activation may also stimulate cAMP formation, since phenylephrine, an α_1-adrenoceptor agonist that does not stimulate the endothelium directly, induces an endothelium-dependent efflux of cAMP from perfused rabbit ear preparations that can be abolished by 18α-GA (Taylor *et al*., 2001). In this situation, however, the magnitude of the pressor response to phenylephrine is unaffected by blockade of gap junctions with 18α-GA, suggesting that endothelium-derived cAMP is unable to modulate constrictor tone in the absence of a co-existent endothelial hyperpolarization that can be transmitted into the media (Taylor *et al*., 2001). An additional pathway that may participate in agonist-induced synthesis of cAMP by the endothelium involves the formation of eicosanoid metabolites of arachidonic acid that stimulate adenylyl cyclase *via* a G protein-dependent mechanism (Node *et al*., 2001; Popp *et al*., 2002; see below).

In rabbit iliac and rat mesenteric arteries, EDHF-type responses are also accompanied by an endothelium-dependent increase in smooth muscle cAMP content that peaks at ~30 s before declining towards control after ~1 min (Taylor *et al*., 2001; Chaytor *et al*., 2002; Griffith *et al*., 2002; Matsumoto *et al*., 2003). This dynamic elevation in smooth muscle cAMP content can be attenuated by blockade of gap junctions with ^{37,43}Gap 27 or 18α-GA, and is therefore a secondary phenomenon, although it is unknown if it is mediated by diffusion of endothelium-derived cAMP through myoendothelial gap junctions, modulation of smooth muscle adenylyl cyclase activity by changes in membrane potential, or the formation of a diffusible intermediate activator of adenylyl cyclase/inhibitor of cAMP phosphodiesterase (Taylor *et al*., 2001; Chaytor *et al*., 2002; Griffith *et al*., 2002). Elevations in smooth muscle cAMP levels have been shown to facilitate electrotonic signalling within the vascular media, and thereby amplify and prolong the transmission of ACh-induced hyperpolarizations to smooth muscle cells remote from the endothelium (Griffith *et al*., 2002). This mechanism is likely to contribute to the marked potentiation of EDHF-type relaxations reported in the presence of IBMX or 8-bromo-cAMP in rabbit iliac arteries (Taylor *et al*., 2001; Chaytor *et al*., 2002; Griffith *et al*., 2002), IBMX in rat mesenteric arteries (Matsumoto *et al*., 2003), and the cell-permeant cAMP analogue Sp-5,6-DCI-cBIMPS in rat renal arteries (Büssemaker *et al*., 2003).

Freely transferable mediators of smooth muscle hyperpolarization

Vasoactive species that are released from the endothelium and have been postulated to contribute to the EDHF phenomenon following diffusion across the extracellular space include K^+ ions, arachidonic acid derivatives (eicosanoids and the endocannabinoid anandamide), H_2O_2 and C-type natriuretic peptide. Controversy nevertheless exists in respect of the nature and physiological relevance of the hyperpolarizing mechanisms activated by such agents and, specifically, whether their ability to increase the open-state probability of smooth muscle K^+ channels accounts for EDHF-type relaxant activity. Although vascular smooth muscle cells express a range of K^+ channels (K_{Ca}, K_{ir}, K_{ATP} and K_v), in the case of the K_{Ca} family, histochemical, patch-clamp and Western blot analysis suggests that the subtype involved in direct smooth muscle hyperpolarization would most likely be BK_{Ca}, because expression of this channel predominates in smooth muscle cells with a contractile phenotype (Neylon *et al*., 1999; Burnham *et al*., 2002). Indeed, there is evidence that functional SK_{Ca} channels are not present in the media of freshly isolated arteries, and that IK_{Ca} channels are preferentially expressed by immature and de-differentiated cultured vascular smooth muscle cells (Neylon *et al*., 1999; Burnham *et al*., 2002). Only a small number of reports have claimed a role for ATP-sensitive K^+ channels (K_{ATP}) channels in the EDHF phenomenon, and studies investigating the role of voltage-gated K^+ channels (K_v) have been inconsistent. The K_v channel inhibitor 4-aminopyridine attenuates EDHF-type responses in rabbit femoral and guinea-pig carotid and coronary arteries, but not in rabbit mesenteric, rat cerebral and human resistance arteries (Murphy & Brayden, 1995; Ohlmann *et al*., 1997; Petersson *et al*., 1997; Eckman *et al*., 1998; Kwon *et al*., 1999; Quignard *et al*., 2000). Notably, 4-AP can also inhibit K_{Ca} channels, and more selective K_v inhibitors, such as dendrotoxin, have not been reported to attenuate the EDHF phenomenon (Adeagbo & Triggle, 1993; Petersson *et al*., 1997; Zygmunt *et al*., 1997a). In rat basilar arteries, 4-AP may also initiate a smooth muscle depolarization that may be conducted electrotonically *via* myoendothelial gap junctions to depress endothelial function, presumably by diminishing the electrochemical gradient for endothelial Ca^{2+} influx (Kamouchi *et al*., 1999; Allen *et al*., 2002).

K^+ ions

K^+ as an EDHF

Exogenous K^+ ions hyperpolarize cell membranes by opening inwardly rectifying, Ba^{2+}-sensitive K^+ channels (K_{ir}) and stimulating a family of Na^+/K^+-ATPase isoenzymes that are constructed from heterogeneous α ion-transporting and β-regulatory subunits, and are inhibited when ouabain binds to the α component of the pump (Blanco & Mercer, 1998; Zaritsky *et al*., 2000). Both hyperpolarizing mechanisms have been suggested to be activated by the efflux of K^+ ions that follow the opening of endothelial K_{Ca} channels by agonists, and thereby contribute to smooth muscle relaxation (Edwards *et al*., 1998). This hypothesis (i.e. that endothelium-derived K^+ is an EDHF) is based on observations that in rat hepatic and mesenteric arteries: (i) EDHF-type responses evoked by ACh can be attenuated by co-administration of Ba^{2+} ions and ouabain, without suppression of the initiating endothelial hyperpolarization, (ii) elevations in extracellular $[K^+]$ hyperpolarize/relax arterial preparations lacking an intact endothelium through mechanisms that can be blocked by ouabain and Ba^{2+}, and (iii) measurements with a K^+-sensitive electrode demonstrate an increase in $[K^+]$ in a putative 'myoendothelial space' following stimulation with ACh (Edwards *et al*., 1998). Functional studies with vessels from K_{ir} channel knockout mice suggest that the Ba^{2+}-sensitive component of the smooth muscle relaxant response to K^+ could involve a K_{ir} 2.1 subtype (Zaritsky *et al*., 2000), and functional studies in concert with specific antibody staining suggest that the ouabain-sensitive component of K^+-induced hyperpolarizations of rat mesenteric arteries involves Na^+/K^+-ATPase isoenzymes possessing α_2 and α_3 subunits that can be inhibited by the glycoside at nanomolar ($\sim$500 nM) concentrations (Blanco & Mercer, 1998; Weston *et al*., 2002). These subunits are stimulated by small elevations in extracellular $[K^+]$ above the concentrations normally employed in organ chamber experiments, that is, 5–6 mM, whereas a more ubiquitously expressed α_1 subunit-containing isoenzyme may be almost fully activated at such levels of $[K^+]_0$ (Blanco & Mercer, 1998; Weston *et al*., 2002).

Reports that EDHF-type responses are often resistant to Ba^{2+} and ouabain in arteries in which exogenous K^+ causes relaxation, even when these inhibitors are administered in combination, nevertheless suggest that endothelium-derived K^+ does not contribute to the EDHF phenomenon in a consistent fashion (Suzuki, 1988; Quignard *et al*., 1999; Doughty *et al*., 2000; 2001; Lacy *et al*., 2000; Coats *et al*., 2001; Coleman *et al*., 2001a, b; McIntyre *et al*., 2001). One potential source of variability is the influence of smooth muscle activation on the ability of endothelium-derived K^+ to serve as an EDHF. Constriction of rat arteries by high concentrations of agonist results in a depolarization that will diminish outward K^+ currents carried by K_{ir} channels, and also promotes an efflux of K^+ *via* smooth muscle K_{Ca} channels, thereby resulting in the creation of an extracellular 'cloud' of K^+ ions that mask the activation of the Na^+/K^+-ATPase by additional sources of K^+ (Dora & Garland, 2001; Richards *et al*., 2001). Indeed, in agonist-constricted arteries, charybdotoxin and iberiotoxin enhance hyperpolarizations and relaxations induced by exogenous K^+ ions by blocking smooth muscle K^+ efflux, in marked contrast to their inhibitory effects against authentic EDHF-type responses to agonists such as ACh (Dora *et al*., 2002; Weston *et al*., 2002). Electrophysiological characterization of the ionic currents activated by agonists and exogenous K^+ in guinea-pig mesenteric arterioles has also cast doubt on the role of K^+ as an EDHF. In these vessels, endothelial and smooth muscle cells behave as an electrical syncytium and exogenous K^+ activates an inwardly rectifying Ba^{2+}-sensitive current, whereas currents evoked by ACh or substance P are outwardly rectifying (Coleman *et al*., 2001a, b). Furthermore, inhibition of basal K_{ir} channel activity with Ba^{2+} causes constriction and depolarization, with subsequent administration of ACh repolarizing the cell membrane by activating channels that are sensitive to charybdotoxin and apamin, and the combination of Ba^{2+} and ouabain failing to modulate the currents evoked by ACh and associated EDHF-type relaxations (Imaeda *et al*., 2000; Coleman *et al*., 2001b).

K^+, ouabain and gap junctions

In some vessels, there is evidence that a component of the relaxation evoked by exogenous K^+ is secondary to activation of endothelial K_{ir} channels, which may be a significant determinant of endothelial membrane potential (Nilius & Droogmans, 2001). In the perfused rat mesenteric bed and rat mesenteric and human subcutaneous arteries, for example, K^+-induced relaxations may be either abolished or attenuated by endothelial denudation, with residual responses remaining sensitive to ouabain, but insensitive to Ba^{2+} (Harris *et al*., 2000; Lacy *et al*., 2000; Dora & Garland, 2001; McIntyre *et al*., 2001). As in the case of agonists, K^+-induced endothelial hyperpolarization can be transmitted to subjacent smooth muscle cells *via* gap junctions, because the endothelium-dependent component of the hyperpolarizing and relaxant response to K^+ is attenuated by ^{37,43}Gap 27 and 18α-GA (Doughty *et al*., 2000; 2001; Richards *et al*., 2001).

Interpretation of the effects of exogenous K^+ and ouabain on vascular tone is also complicated by the ability of the glycoside to impair the functionality of gap junctions and the expression of connexin proteins in a sequential fashion (Schirrmacher *et al*., 1996; Harris *et al*., 2000; Martin *et al*., 2003). This biphasic action correlates with the affinity of ouabain to bind to the dominant Na^+/K^+-ATPase α subunits expressed in different target cells, although it is unknown if this reflects altered Na^+/K^+ homeostasis or conversion of the Na^+/K^+-ATPase into a general signal transducer that modulates downstream signal transduction pathways (Martin *et al*., 2003). In COS fibroblasts and HeLa epithelial cells, whose endogenous α_1 subunits possess high ouabain affinity ($K_i \sim 0.3\,\mu$M), low concentrations (0.1–10 μM) attenuate dye transfer within $\sim$1 h, whereas high concentrations (100 μM–1 mM) are required to achieve an equivalent effect in cultured rat aortic A7r5 myocytes or COS cells selected to express the ouabain-resistant rodent α_1 Na^+/K^+-ATPase subunit ($K_i \sim 100\,\mu$M) (Martin *et al*., 2003). More prolonged exposure of A7r5 cells to ouabain, at concentrations that inhibit the resistant rodent α_1 subunit, further results in a time-dependent loss of endogenous Cx40 and Cx43 protein, which is first evident after 90 min, almost complete after 4 h, but reversed on drug washout (Martin *et al*., 2003). Observations that high concentrations of ouabain (100 μM–1 mM) are required to attenuate EDHF-type relaxations in rat mesenteric, gastric, renal and femoral arteries, whereas nanomolar concentrations

inhibit the α_2 and α_3 subunits that mediate hyperpolarization (Doughty *et al*., 2000; Van de Voorde & Vanheel, 2000; Jiang *et al*., 2001; Weston *et al*., 2002; Savage *et al*., 2003), thus suggest that the mechanisms mediating agonist- and K^+-induced smooth muscle hyperpolarizations are distinct, and that effects of ouabain against gap junctional communication might contribute to its ability to inhibit the EDHF phenomenon in the rat vasculature.

Time-dependent suppression of connexin expression by ouabain might also explain observations by Nelli *et al.* (2003) that the glycoside fails to block bradykinin-induced EDHF-type relaxations of bovine coronary arteries after 30 min, whereas relaxation is abolished after 90 min. Indeed, these authors have suggested that inappropriately short exposure times might account for the highly variable effects of ouabain against EDHF-type relaxations of bovine and porcine arteries reported in the literature (Drummond *et al*., 2000; Pratt *et al*., 2001; Büssemaker *et al*., 2002; Nelli *et al*., 2003). A case can therefore be made for systematic examination of the concentration- and time-dependent effects of ouabain in vessels in which the glycoside has been reported to be ineffective as an inhibitor of the EDHF phenomenon (Suzuki, 1988; Quignard *et al*., 1999; Doughty *et al*., 2000; 2001; Lacy *et al*., 2000; Coats *et al*., 2001; Jiang & Dusting, 2001; McIntyre *et al*., 2001). It also remains to be determined if the ability of glycyrrhetinic acid derivatives to inhibit the Na^+/K^+-ATPase, which could in theory reflect the similarity of their steroidal structure to that of ouabain, contributes to their action against gap junctional communication, although such compounds are reportedly $\sim$100-fold less potent as inhibitors of the ionic activity of the pump than ouabain (Terasawa *et al*., 1992).

Arachidonate metabolites

Evidence that mobilization of arachidonic acid from membrane phospholipids by phospholipase A_2 (PLA_2) may be an initiating step in the EDHF phenomenon has been obtained in a range of arteries and vascular beds in which inhibitors of this enzyme attenuate NO/prostanoid-independent relaxations (Table 2). Observations that inhibitors directed against either the cytosolic Ca^{2+}-dependent ($cPLA_2$) or secretory ($sPLA_2$) forms of the enzyme are capable of depressing EDHF-type relaxations could reflect physiological cross-talk between these isoenzymes (Balsinde *et al*., 2002), as well as a potential lack of pharmacological selectivity (Table 2). The participation of arachidonate metabolites in the EDHF phenomenon is also suggested by similarities in the endothelium-dependent effects of agonists and mellitin, a polypeptide that activates PLA_2 through a receptor-independent mechanism and evokes EDHF-type responses that are attenuated by inhibition of $cPLA_2$ or blockade of gap junctional communication by ^{37,43}Gap 27 in the rabbit superior mesenteric artery (Hutcheson *et al*., 1999; Hutcheson & Griffith, 2000). In theory, the ability of PLA_2 inhibitors to depress the EDHF phenomenon could reflect impaired synthesis of arachidonate products that induce smooth muscle hyperpolarization through a paracrine action or, alternatively, impaired synthesis of products that promote endothelial hyperpolarization by stimulating Ca^{2+} influx *via* store-operated Ca^{2+} channels (Graier *et al*., 1995; Fleming, 2001; see below). Observations that mobilization of arachidonate by agonists or SERCA inhibitors is itself crucially dependent on sustained endothelial Ca^{2+} influx *via* store-operated channels, and therefore suppressed by inhibitors of the Ca^{2+}-dependent $cPLA_2$ or buffering Ca^{2+} influx with an intracellular Ca^{2+} chelator (Millanvoye-Van Brussel *et al*., 1999), may explain why a reportedly selective inhibitor (HELSS) of the Ca^{2+}-independent cytosolic isoenzyme ($iPLA_2$) is inactive against EDHF-type relaxations (Table 2).

Eicosanoids

The endothelium is capable of synthesizing four epoxyeicosatrienoic acid regioisomers (5,6-, 8,9-, 11,12- and 14,15-EET) from arachidonic acid *via* cytochrome P_{450} (CYP_{450}) epoxygenases, variously reported as belonging to the CYP 1A, 2C and 2J subfamilies, and complementary experimental approaches have suggested that these eicosanoids can contribute

Table 2 Reported effects of three classes of PLA_2 inhibitor against EDHF-type relaxations

PLA_2 isoform	*$cPLA_2$*	*$iPLA_2$*	*$sPLA_2$*	*Inhibits EDHF/species and artery type*	*Reference*
ONO-RS-082	+		+	+ Rabbit mesenteric	Hutcheson *et al.* (1999)
				+ Rat coronary	Fulton *et al.* (1996)
				− Rat mesenteric	Tanaka *et al.* (1999)
$AACOCF_3$	++	+		+ Rabbit mesenteric	Hutcheson *et al.* (1999); Hutcheson & Griffith (2000)
				+ Rat coronary, mesenteric, cerebral	Fulton *et al.* (1996); Adeagbo & Henzel (1998); You *et al.* (2002)
				− Guinea-pig coronary, carotid − bovine coronary	Yamanaka *et al.* (1998); Quignard *et al.* (2002); Drummond *et al.* (2000)
$PACOCF_3$	+	++	+	+ Rat cerebral	You *et al.* (2002)
OOPC			+	+ Human subcutaneous	Coats *et al.* (2001)
				+ Rat coronary, mesenteric	Fulton *et al.* (1996); Adeagbo & Henzel (1998)
HELSS		+		− Rabbit mesenteric	Hutcheson *et al.* (1999)
				− Rat mesenteric, cerebral	Adeagbo and Henzel (1998); You *et al.* (2002)

+/− signs reflect the ability of each compound to inhibit different PLA_2 isoenzymes and relaxation. $cPLA_2$, cytosolic Ca^{2+}-dependent phospholipase A_2; $iPLA_2$, cytosolic Ca^{2+}-independent phospholipase A_2; $sPLA_2$, secretory phospholipase A_2. ONO-RS-082, 2-(*p*-amylcinnamoyl) amino-4-chlorobenzoic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; $PACOCF_3$, palmitoyl trifluoromethyl ketone; OOPC, oleyloxyethyl phosphorylcholine; HELSS, haloenol lactone suicide substrate.

to the EDHF phenomenon (Campbell & Harder, 1999; Fleming, 2001; Node *et al*., 2001). In some vessels, CYP_{450} inhibitors, such as clotrimazole, sulfaphenazole and ODYA, and an EET antagonist (14,15-EEZE) impair EDHF-type relaxations and hyperpolarizations, and in the converse sense, compounds that upregulate the expression of CYP_{450} epoxygenases, such as nifedipine and β-napthoflavone, can enhance EET synthesis and potentiate EDHF-type responses (Campbell & Harder, 1999; Fisslthaler *et al*., 1999; 2000; Fleming, 2001; Gauthier *et al*., 2002). Although some of these pharmacological probes exert nonspecific actions, antisense oligonucleotides targeted to the coding region of CYP 2C attenuate EDHF-type responses in porcine coronary arteries and hamster skeletal muscle arterioles, consistent with the involvement of this isoenzyme in events that lead to relaxation, at least in specific vessels (Fisslthaler *et al*., 1999; Bolz *et al*., 2000).

Smooth muscle action One mechanism that may explain these observations is that EETs act as freely transferable EDHFs, since in some arteries they activate smooth muscle BK_{Ca} channels, possibly *via* the occupation of specific membrane receptors (Hecker *et al*., 1994; Campbell *et al*., 1996; Fisslthaler *et al*., 1999; Gauthier *et al*., 2002; Snyder *et al*., 2002; Archer *et al*., 2003). In canine coronary microvessels, exogenous EETs are reportedly capable of evoking relaxation at subnanomolar concentrations, and cascade bioassay studies using bovine coronary arteries or cultured porcine coronary endothelium as the donor tissue have detected release of an EDHF whose effects on BK_{Ca} channel activity in downstream vascular myocytes can be mimicked by exogenous 5,6-EET or 14,15-EET (Popp *et al*., 1996; Gebremedhin *et al*., 1998; Oltman *et al*., 1998). However, in porcine coronary and human internal mammary arteries, in which 11,12-EET is the dominant regioisomer produced by the endothelium, concentrations of exogenous 11,12-EET in the range 0.1–3 μM are required to evoke iberiotoxin-sensitive smooth muscle relaxations that mimic the 'authentic' EDHF-type response to agonists, and it remains unproven whether the endothelium of these arteries can generate this eicosanoid at equivalent levels (Fisslthaler *et al*., 1999; 2000; Archer *et al*., 2003).

Endothelial actions EETs may also exert autocrine effects that promote endothelial hyperpolarization through mechanisms that are functionally indistinguishable from those initiated by agonists. In the rabbit superior mesenteric artery, for example, exogenous 5,6-EET is devoid of direct smooth muscle relaxant activity, but evokes EDHF-type relaxations in preparations with intact endothelium (Hutcheson *et al*., 1999). Analogously, in the porcine coronary artery, exogenous 11,12-EET induces EDHF-type smooth muscle hyperpolarizations that are sensitive to the combination of apamin and charybdotoxin, with only a small additional direct BK_{Ca}-mediated change in smooth muscle membrane potential being evident (Edwards *et al*., 2000). Notably, in cultured human, porcine and bovine endothelial cells, nanomolar concentrations of 5,6-EET elevate endothelial Ca^{2+} levels by promoting Ca^{2+} entry *via* store-operated channels, leading to the suggestion that this regioisomer is an endothelial 'Ca^{2+} influx factor' whose synthesis by CYP_{450} epoxygenase is activated by the store depletion associated with administration of agonists or SERCA inhibitors (Graier *et al*., 1995; Hoebel *et al*., 1997; Rzigalinski *et al*., 1999; Xie *et al*., 2002). EET-induced elevations in $[Ca^{2+}]_i$ would be expected to increase the open-state probability of all endothelial K_{Ca} subtypes, and there is also evidence that each of the four regioisomers can open BK_{Ca} channels directly when applied to the cytosolic side of the plasmalemma in cultured endothelial cells (Baron *et al*., 1997).

Further similarities in the endothelial effects of EETs, agonists and SERCA inhibitors are evident in rabbit mesenteric and iliac arteries in which EDHF-type relaxations evoked by exogenous 5,6-EET mimic the response to ACh and cyclopiazonic acid as they are attenuated by ^{37,43}Gap 27, 18α-GA and 2′,5′-ddA, and therefore involve gap junctional communication and formation of cAMP (Hutcheson *et al*., 1999; Taylor *et al*., 2001). Although the ability of 5,6-EET to elevate endothelial $[Ca^{2+}]_i$ may promote the formation of cAMP by Ca^{2+}-stimulated adenylyl cyclase isoforms (*see above*), all the four EET regioisomers may also enhance endothelial cAMP synthesis *via* activation of adenylyl cyclase by $G_{\alpha s}$ following ADP ribosylation of this component of the heterotrimeric G protein complex (Li *et al*., 1999; Node *et al*., 2001). Evidence for a functional role of this pathway has been obtained in cultured porcine coronary endothelial cells, in which bradykinin enhances inter-endothelial dye transfer through a cAMP-dependent mechanism that may be secondary to endogenous synthesis of 11,12-EET (Popp *et al*., 2002). The ability of bradykinin and 11,12-EET to modulate the functionality of gap junctions in these cells is biphasic, and consists of an initial rapid enhancement of inter-endothelial communication, followed by a delayed reduction in dye transfer after 5–10 min that results from phosphorylation of Cx43 by extracellular regulated kinases 1/2 (ERK1/2) (Brandes *et al*., 2002; Popp *et al*., 2002).

Evidence against the involvement of EETs There is nevertheless substantial evidence to suggest that endogenous synthesis of EETs cannot be regarded as a universal participant in the EDHF phenomenon. Inhibitors of PLA_2 are inactive in arteries from some species (Table 2), and more specifically, in rat mesenteric and hepatic arteries, guinea-pig carotid arteries, bovine coronary arteries and the perfused mouse hindlimb EDHF-type responses are unaffected by blockade of CYP_{450} epoxygenases (Zygmunt *et al*., 1996; Fukao *et al*., 1997b; Vanheel & Van de Voorde, 1997; Chataigneau *et al*., 1998a; Brandes *et al*., 2000; Drummond *et al*., 2000; Quignard *et al*., 2002). Paracrine activation of smooth muscle BK_{Ca} channels by endogenously released EETs also seems improbable in the guinea-pig coronary artery, in which iberiotoxin attenuates smooth muscle hyperpolarizations evoked by exogenous 11,12-EET, whereas in this and other guinea-pig arteries authentic EDHF-type responses to ACh are unaffected by the toxin (Petersson *et al*., 1997; Eckman *et al*., 1998; Yamanaka *et al*., 1998). In guinea-pig carotid arteries, exogenous EETs also fail to promote hyperpolarization and mediate relaxation in preparations with intact endothelium, thus seemingly excluding autocrine effects (Chataigneau *et al*., 1998a). As noted above, EDHF-type relaxations may also be insensitive to iberiotoxin in rat arteries (Rapacon *et al*., 1996; Plane *et al*., 1997; Edwards *et al*., 1998).

Anandamide

Another endothelial derivative of arachidonic acid that has been proposed as an EDHF is the endocannabinoid

N-arachidonylethanolamide (anandamide), which can evoke smooth muscle hyperpolarizations and relaxations that are susceptible to BK_{Ca} channel blockade by iberiotoxin (Randall *et al*., 1996; Deutsch *et al*., 1997; Plane *et al*., 1997). Observations that the CB_1 cannabinoid receptor antagonist SR141716A attenuates agonist-evoked EDHF-type relaxations initially suggested a role for the activation of specific receptors following endogenous release of anandamide (Randall *et al*., 1996; White & Hiley, 1997). It subsequently became apparent, however, that anandamide is unlikely to be a universal participant in the EDHF phenomenon, as it relaxes endothelium-denuded rat hepatic arteries without altering smooth muscle membrane potential, and fails to evoke hyperpolarization and relaxation in endothelium-denuded porcine coronary arteries, which nevertheless exhibit prominent EDHF-type responses (Zygmunt *et al*., 1997b; Chataigneau *et al*., 1998b). Furthermore, in some arteries, low micromolar concentrations of SR141716A, which are thought to be selective for the CB_1 receptor subtype, fail to inhibit EDHF-type relaxations evoked by muscarinic agonists, bradykinin, and even exogenous anandamide itself (Plane *et al*, 1997; White & Hiley, 1997; Zygmunt *et al*, 1997b; Chataigneau *et al*., 1998b; Fulton & Quilley, 1998; Pratt *et al*., 1998; Wagner *et al*., 1999). CB_1 receptor agonists may also be without EDHF-type vasodilator activity *in vivo*, for example, in the rabbit under conditions of combined NO synthase and cyclooxygenase blockade (Niederhoffer & Szabo, 1999).

In addition to direct smooth muscle relaxant activity, in rat hepatic and rabbit mesenteric arteries, exogenous anandamide can itself stimulate endothelium-dependent relaxations that are independent of NO and prostanoids, and thus exhibit the characteristics of the EDHF phenomenon (Zygmunt *et al*., 1997b; Chaytor *et al*., 1999). One explanation might be that anandamide can occupy endothelial CB_2 receptors and mobilize Ca^{2+} from intracellular stores *via* the formation of $InsP_3$, thereby activating Ca^{2+} capacitative entry (Zoratti *et al*., 2003). However, in rabbit mesenteric arteries, EDHF-type relaxations evoked by anandamide, while being insensitive to the selective CB_1 receptor antagonist LY320135, appear to involve facilitated endothelial uptake of the endocannabinoid *via* the high-affinity anandamide transporter (Chaytor *et al*., 1999). In rabbit mesenteric arteries, observations that ^{37,43}Gap 27 and 18α-GA and high micromolar concentrations of SR141716A each block EDHF-type relaxations evoked by anandamide and ACh may reflect a common ability to attenuate electrotonic signalling, as dye-transfer studies have demonstrated that SR141716A can 'nonspecifically' block gap junctional communication (Chaytor *et al*., 1999). High micromolar concentrations of SR141716A may also impair capacitative Ca^{2+} influx initiated by agonists and SERCA inhibitors (Mombouli *et al*., 1999).

Hydrogen peroxide

In vitro and *in vivo* studies have indicated that endogenous formation of H_2O_2 may contribute to aspects of circulatory control as diverse as the vascular myogenic response and coronary autoregulation (Nowicki *et al*., 2001; Yada *et al*., 2003). H_2O_2 has also been implicated as an EDHF in isolated human and mouse mesenteric arteries, on the basis that NO/prostanoid-independent hyperpolarizations and relaxations to ACh are attenuated by catalase and associated with endothelial production of H_2O_2, as assessed by imaging with the oxidant-sensitive dye dihydrodichlorofluorescein (Matoba *et al*., 2000; 2002; Rabelo *et al*., 2003). Catalase also attenuates EDHF-type relaxations evoked by bradykinin and a cytokine (leukaemia inhibitory factor) in rat mesenteric and porcine pial arteries (Kimura *et al*., 2002; Lacza *et al*., 2002). By contrast, EDHF-type relaxations of human radial arteries, which account for ~50% of the relaxant response to ACh in this vessel, are insensitive to catalase (Hamilton *et al*., 2001), and the enzyme also fails to impair EDHF-type responses in porcine coronary arteries and the perfused bovine ocular and rat and mouse mesenteric vascular beds (Bény & Von der Weid, 1991; Pomposiello *et al*., 1999; Brandes *et al*., 2000; McNeish *et al*., 2002).

The reasons for such marked vessel and species differences remain unclear, although it has become apparent that the cellular mechanisms that mediate the smooth muscle response to H_2O_2 exhibit considerable heterogeneity. Indeed, in rat mesenteric arteries, the response to exogenous H_2O_2 may be biphasic, with low concentrations promoting constriction (Gao *et al*., 2003). Furthermore, in human, rat, murine and porcine arteries, hyperpolarizations and relaxations evoked by exogenous H_2O_2 display widely different susceptibilities to the pharmacological blockade of K_{Ca}, K_{ATP} and K_v channels and the Na^+/K^+-ATPase, and catalase-sensitive 'EDHF-type' relaxations exhibit differential sensitivities to K_{Ca} and K_{ATP} channel blockers in human, murine and rat arteries compared to porcine arteries (Pomposiello *et al*., 1999; Barlow *et al*., 2000; Matoba *et al*., 2000; 2002; Kimura *et al*., 2002; Lacza *et al*., 2002; Gao *et al*., 2003; Miura *et al*., 2003). In the rabbit, endothelium-derived H_2O_2 may more correctly be regarded as a relaxing factor, rather than an EDHF, that depresses smooth muscle tone by modulating the sensitivity of the contractile apparatus to Ca^{2+}, rather than causing changes in membrane potential (Iesaki *et al*., 1996; Chaytor *et al*., 2003; Itoh *et al*., 2003). In rabbit iliac arteries, for example, the Ca^{2+} ionophore A23187 evokes a pronounced release of H_2O_2 from the endothelium that causes large catalase-sensitive relaxations that are independent of changes in smooth muscle membrane potential, and can be clearly distinguished from a co-existent catalase-insensitive conducted hyperpolarization that is abolished by connexin-mimetic peptides (Chaytor *et al*., 2002; 2003). The action of A23187 in this vessel contrasts with ACh, which only weakly stimulates endothelial H_2O_2 production and evokes EDHF-type smooth muscle hyperpolarizations and relaxations that are essentially unaffected by catalase (Chaytor *et al*., 2003). There is also evidence that H_2O_2 may act synergistically with electronically conducted mechanisms in human mesenteric arteries, in which EDHF-type relaxations and hyperpolarizations evoked by bradykinin are incompletely attenuated by catalase, and a gap junction-dependent component is revealed by administration of 18α-GA (Matoba *et al*., 2002).

A further difficulty in classifying H_2O_2 unambiguously as an EDHF is that electrochemical measurements of endogenous H_2O_2 production by the endothelium reveal extracellular levels of just 10–100 nM following stimulation with A23187, for example, in the rat aorta (Cosentino *et al*., 1998), whereas in a variety of human, rat, murine, rabbit and porcine arteries, authentic H_2O_2 induces significant direct smooth muscle hyperpolarization/relaxation only at 'supraphysiological' concentrations in the range 100 μM–1 mM (Bény & Von der Weid, 1991; Karasu, 1999; Matoba *et al*., 2000; Fujimoto *et al*., 2001; Chaytor *et al*., 2003; Gao *et al*., 2003; Hattori *et al*., 2003;

Miura *et al*., 2003). Speculatively, this discrepancy between the functional effects of endogenous and authentic H_2O_2 might reflect higher biological activity of 'nascent' H_2O_2 generated enzymatically in close proximity to its site of action (Chaytor *et al*., 2003; Miura *et al*., 2003). The enzymatic mechanisms that underpin production of H_2O_2 by the endothelium are also controversial. Matoba *et al*. (2000) have suggested that agonist-induced formation of H_2O_2 depends on dismutation of eNOS-generated superoxide anions, on the basis that ACh-evoked hyperpolarization, relaxation and endothelial dihydrodichlorofluorescein fluorescence are depressed in mesenteric arteries from eNOS knockout mice. This hypothesis would, however, appear to conflict with evidence that pharmacological inhibition of eNOS by L-arginine analogues causes a reduction in the formation of superoxide anions and H_2O_2 by this enzyme, and that release of H_2O_2 can still be detected in the presence of such inhibitors (Cosentino *et al*., 1998; Xia *et al*., 1998; Chaytor *et al*., 2003). Indeed, in human resistance arteries, catalase-sensitive endothelium-dependent dilatations induced by fluid shear stress have been attributed to H_2O_2 generated by Complex I of the mitochondrial electron transport chain (Liu *et al*., 2003), and in cultured bovine aortic endothelial cells flow-induced H_2O_2 formation appears to originate from xanthine oxidase (McNally *et al*., 2003). It also remains to be determined if autocrine effects of H_2O_2 within the endothelium contribute to the EDHF phenomenon, since this reactive oxygen species can activate endothelial PLA_2 and elevate endothelial $[Ca^{2+}]_i$ (Boyer *et al*., 1995; Saito *et al*., 2001).

C-type natriuretic peptide

Endothelial cells are capable of synthesizing and storing C-type natriuretic peptide, which is structurally related to atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). CNP can subsequently be released by endothelium-dependent agonists, such as ACh and bradykinin, to activate specific natriuretic peptide receptors (NPR) on vascular smooth muscle cells and thereby modulate arterial tone (Wennberg *et al*., 1999). Following occupation of the NPR-B subtype, which is coupled to the particulate guanylyl cyclase enzyme, CNP promotes relaxation through an NO-independent elevation in cGMP levels (Tao *et al*., 1995; Mori *et al*., 1997; Barber *et al*., 1998). Since CNP-induced relaxation has variously been associated with an opening of K_{Ca} or K_{ATP} channels that may be secondary to cGMP-dependent channel phosphorylation, the peptide may thus be considered as an EDHF (Barber *et al*., 1998; Honig *et al*., 2001). It has also been suggested that CNP can activate a non-guanylyl cyclase-coupled NPR-C receptor that opens K_{ir} channels through a pertussis toxin-sensitive G protein-linked mechanism that contributes to EDHF-type relaxations in rat mesenteric arteries (Chauhan *et al*., 2003a). The generality of this mechanism, nevertheless, remains to be established since pertussis toxin does not consistently attenuate EDHF-type relaxations (Graier *et al*., 1996), and such relaxations are not associated with the elevations in cGMP levels that typically follow administration of CNP. Indeed, Barton *et al*. (1998) concluded that CNP is not the principal mediator of the EDHF phenomenon in porcine coronary arteries, on the basis that the relaxation and the associated hyperpolarization evoked by exogenous CNP were much less pronounced than those evoked by bradykinin.

Interactions with nitric oxide

Agonist-induced responses

Some studies have suggested that NO may contribute to agonist-induced EDHF-type responses, either as a consequence of 'residual' activity resulting from incomplete pharmacological blockade of eNOS or release of NO from preformed endothelial stores (Cohen *et al*., 1997; Chauhan *et al*., 2003b). While it is apparent that NO can hyperpolarize vascular smooth muscle cells by opening K^+ channels, the ambient NO levels necessary to evoke changes in membrane potential are nevertheless generally much higher than those required to induce relaxation (Parkington *et al*., 1995; Tare *et al*., 2000). Furthermore, the existence of a distinct hyperpolarizing mechanism, activated either by agonists or fluid shear stress, is evident in eNOS knockout mice (Waldron *et al*., 1999; Brandes *et al*., 2000; Huang *et al*., 2000; 2001). There is also accumulating evidence to support a dynamic reciprocal relationship between EDHF-type and NO-mediated mechanisms of vasomotor control: EDHF-type responses are potentiated by pharmacological blockade or knockout of eNOS, and reduced by exogenous NO or the overproduction of NO that occurs in sepsis (Bauersachs *et al*., 1996; McCulloch *et al*., 1997; Waldron *et al*., 1999; Hutcheson *et al*., 1999; Kessler *et al*., 1999; Huang *et al*., 2000; 2001; Hutcheson & Griffith, 2000). Indeed, in the canine coronary microcirculation, the EDHF phenomenon can completely offset loss of NO-mediated vasodilatation in respect of the dilator response to bradykinin (Nishikawa *et al*., 2000), with the corollary that EDHF-type relaxations can serve as a 'back-up' mechanism that in specific situations may fully compensate for diminished NO activity *in vivo*. Mechanisms contributing to the inhibitory action of NO against the EDHF phenomenon may involve direct effects of NO or its second messenger cGMP (Olmos *et al*., 1995; McCulloch *et al*., 1997). Specifically, these may include: (i) inhibition of CCE into endothelial cells following cGMP-dependent, protein kinase G (PKG)-mediated phosphorylation of the store-operated channels that mediate Ca^{2+} influx (Dora *et al*., 2001), (ii) attenuated CCE as a consequence of enhanced SERCA-mediated filling of Ca^{2+} stores following phosphorylation of phospholamban by PKG (Trepakova *et al*., 1999; Mundina-Weilenmann *et al*., 2000) or cGMP-independent, NO-mediated S-glutathiolation of the SERCA pump (Adachi *et al*., 2002), and (iii) inhibitory effects of NO on the activity of CYP_{450} epoxygenases (Fleming, 2001). Although there is evidence that NO can reduce the permeability of gap junctions in neurones and retinal cells, probably *via* PKG-mediated phosphorylation of connexin proteins (Lu & McMahon, 1997; Strata *et al*., 1998; Xin & Bloomfield, 2000), it remains to be established if a similar mechanism modulates electrotonic conduction in the vascular wall.

As vessel size diminishes in successive branch generations of many microvascular beds, including the human myocardium, so the magnitude of EDHF-type relaxations/dilations increases relative to NO-mediated responses (Hwa *et al*., 1994; Shimokawa *et al*., 1996; Berman & Griffith, 1997; 1998; Miura *et al*., 1999; Tomioka *et al*., 1999). More specifically, there is an inverse correlation between the magnitude of NO- and gap junction-dependent relaxations evoked by ACh in rabbit resistance arteries of different sizes (Berman *et al*., 2002). While such observations could reflect the suppression of

EDHF-type responses by NO, through the mechanisms noted above, other factors may also contribute to spatial heterogeneity in the contribution of gap junctions to relaxation. For example, the frequency of points of focal contact between endothelial and smooth muscle cells (Aydin *et al*., 1991; Kristek & Gerova, 1992), and more specifically myoendothelial gap junction plaques (Sandow & Hill, 2000), is most numerous in distal vessels, suggesting that there may be closer electrical coupling between the two cell layers as vessel size diminishes, with obvious implications for electrotonic signalling. The larger EDHF-type relaxations observed in distal vessels could also reflect regional variability in the cellular mechanisms that maintain contraction: in small arteries, force development is highly dependent on Ca^{2+} influx *via* voltage-dependent channels (Tomioka *et al*., 1999), and would therefore be expected to be particularly sensitive to hyperpolarizing signals conducted *via* gap junctions.

Shear stress-induced responses

A major physiological role of the endothelium is to optimize tissue perfusion through an adaptive dynamic flow-dependent mechanism that normalizes intimal shear stress following changes in blood flow and minimizes the hydraulic work expended in the maintenance of flow (see Griffith (2002) for a review). It is well-established that the underlying cellular mechanisms may involve endothelial NO production by shear forces, and there is emerging evidence also for a contribution from EDHF-type mechanisms, since flow-dependent dilations of porcine coronary and rat mesenteric arteries and mouse skeletal muscle arterioles are attenuated by high extracellular $[K^+]$ and/or blockade of K_{Ca} channels (Takamura *et al*., 1999; Dube & Canty, 2001; Huang *et al*., 2001). Evidence that increases in intimal shear forces induce endothelial hyperpolarization has been provided by observations that flow activates endothelial K_{ir} channels and that K_{Ca} channel blockade with apamin/charybdotoxin depresses flow-induced NO release (Olesen *et al*., 1988; Hutcheson & Griffith, 1994), although it has yet to be demonstrated experimentally that shear-induced endothelial hyperpolarization is conducted electrotonically into the media *via* gap junctions. A further parallel between agonist and shear-induced responses is reflected by the existence of a reciprocal relationship between NO-mediated and EDHF-type mechanisms of vasodilation that is evident in respect of flow-dependent dilation: normally mediated by co-release of NO and prostanoids, an EDHF-type component is unmasked by inhibitors of eNOS and cyclooxygenase in arterioles from wild-type mice, and can also substitute for the loss of NO in vessels from eNOS knockouts (Huang *et al*., 2000; 2001). A role for EETs in flow-dependent dilation has also been proposed in such knockout animals (Huang *et al*., 2001).

Conclusions and future directions

Diverse and often conflicting reports in the literature suggest that pathways involved in the EDHF phenomenon can be vessel-, species- and, in some instances, even laboratory-specific. There is nevertheless growing evidence that the two central components of this dilatory mechanism are an initiating endothelial hyperpolarization that depends on the opening of K_{Ca} channels, and its subsequent electrotonic relay through the vascular wall *via* myoendothelial and homocellular smooth muscle gap junctions. This contrasts with the idea that EDHF-type relaxations are mediated by a freely transferable factor that activates smooth muscle K_{Ca} channels (such as EETs or H_2O_2). Indeed, it can be argued that such mechanisms are mutually antagonistic, because passive spread of endothelial hyperpolarization would be expected to promote the *closure* of K_{Ca} channels, whose open-state probability will be reduced by the decrease in smooth muscle $[Ca^{2+}]_i$ known to accompany the EDHF phenomenon.

Capacitative Ca^{2+} entry (CCE) may not only sustain the K_{Ca} channel opening that underpins endothelial hyperpolarization, but also promote a prostanoid-independent synthesis of cAMP that facilitates electrotonic spread of this electrical response through the vessel wall *via* gap junctions. Further research is necessary to clarify the role of eicosanoids in the EDHF phenomenon, since EETs can exert autocrine effects within the endothelium, including the ability to stimulate CCE at nanomolar concentrations, to promote membrane hyperpolarization by opening endothelial K_{Ca} channels, and to stimulate cAMP synthesis. Interactions between these mechanisms may provide the basis for a unifying hypothesis that accounts for many characteristics of the EDHF phenomenon, at least in terms of the signalling events that occur in the endothelium of some arteries (Figure 3). It should nevertheless be noted that the role of arachidonate metabolites is unlikely to be universal, because in a significant number of reports inhibitors of PLA_2 or CYP_{450} epoxygenase fail to attenuate NO/prostanoid-independent relaxations. Indeed, even in species in which exogenous EETs are capable of evoking EDHF-type relaxations that involve gap junctional communication and cAMP synthesis, such as the rabbit, the endothelium may normally be unable to synthesize these regioisomers at physiologically active concentrations (Pfister *et al*., 1991). K^+ ions, which can promote smooth muscle relaxation by stimulating Na^+/K^+-ATPases and K_{ir} channels, may be relevant to the EDHF phenomenon only when physiological arterial tone is low, since contraction is accompanied by an efflux of K^+ ions from smooth muscle cells that can mask the ability of endothelium-derived K^+ to promote smooth muscle hyperpolarization. The participation of K^+ is also complicated by an ability to hyperpolarize the endothelium by activating K_{ir} channels, thereby initiating an endothelial hyperpolarization that can be conducted into the media *via* gap junctions, although a physiological role for this mechanism has yet to be established. The involvement of endothelium-derived H_2O_2 in NO- and prostanoid-independent relaxations also requires further evaluation as this reactive oxygen species can variably activate smooth muscle K_{Ca} and K_{ATP} channels, the Na^+/K^+-ATPase, and also modulate the sensitivity of the smooth muscle contractile machinery to Ca^{2+} ions independently of changes in membrane potential.

From a physiological perspective, the role of the EDHF phenomenon in integrative vascular control may be of particular importance in the microcirculation, where there may be a general inverse relationship between the amplitude of gap junction-dependent and NO-mediated responses. Complementary contributions for these two dilatory mechanisms are implicit in their relative distribution from larger to smaller arteries and in their potential for compensatory adaptation, and may ultimately provide a novel focus for understanding circulatory

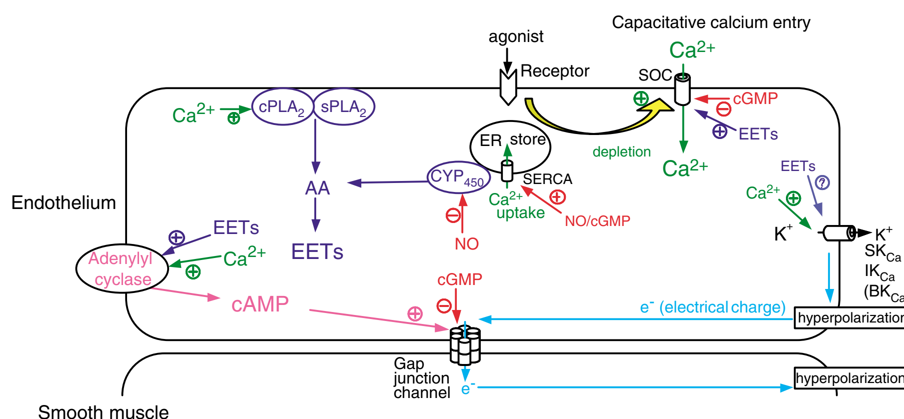


Figure 3 Interactions within the endothelium that may participate in the EDHF phenomenon and involve a complex matrix of pathways that link electrotonic signalling with Ca^{2+} homeostasis, cAMP accumulation and arachidonate metabolism. The initiating endothelial hyperpolarization is dependent on the opening of Ca^{2+}-activated K^+ channels (K_{Ca}) by elevations in cytosolic free Ca^{2+} that result principally from capacitative Ca^{2+} entry *via* store-operated channels (SOC). This influx is triggered by depletion of Ca^{2+} stores in the endoplasmic reticulum. Hyperpolarization is conducted to subjacent smooth muscle cells *via* myoendothelial gap junctions, and may subsequently spread through the vessel wall *via* homocellular gap junctions that couple smooth muscle cells (not shown). Elevations in cytosolic Ca^{2+} may also elevate cAMP levels, thereby enhancing the electrical conductance of myoendothelial gap junctions. Epoxyeicosatrienoic acid (EET) metabolites of arachidonic acid (AA) may contribute to endothelial hyperpolarization by opening SOCs and elevating $[Ca^{2+}]_i$ as well as possible direct effects on K_{Ca} channels. EETs may also stimulate the synthesis of cAMP by adenylyl cyclase following ADP ribosylation by $G_{\alpha s}$. The sites at which NO and its second messenger cGMP could theoretically inhibit endothelial and smooth muscle hyperpolarization are shown in red (see text). Note that redundancy in these interacting pathways will still allow the endothelium to mediate EDHF-type relaxations in arteries in which arachidonic acid metabolism does not contribute to Ca^{2+} homeostasis through autocrine mechanisms. $cPLA_2$, cytosolic Ca^{2+}-dependent phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; ER, endoplasmic reticulum; CYP P_{450}, cytochrome P_{450} epoxygenase; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase.

regulation in disease states where NO bioavailability is decreased and 'EDHF' upregulated. These range from atheroma and its risk markers such as hypertension and diabetes, to heart failure and ischaemia/reperfusion injury (Katz & Krum, 2001; Wigg *et al*., 2001; Marrelli, 2002; Sofola *et al*., 2002). In rats rendered hypertensive by a high-salt diet, for example, apparently 'normal' endothelial-dependent responses to ACh may conceal major alterations in the relative contributions of NO-mediated and EDHF-type mechanisms to relaxation (Sofola *et al*., 2002), and in spontaneously hypertensive rats enhanced myoendothelial coupling can compensate for hyperplastic structural changes in the media, thereby maintaining a functional role for the EDHF phenomenon (Sandow *et al*., 2003). In diabetic rats, reductions in the amplitude of EDHF-type relaxations may reflect cAMP phosphodiesterase overactivity (specifically of the PDE3 isoenzyme), with enhanced cAMP hydrolysis presumably resulting in impaired gap junctional communication, so that relaxation can be normalized by pharmacological inhibition of PDE3 (Matsumoto *et al*., 2003). EDHF-type responses are also enhanced in arteries from hyperthyroid rats as a consequence of upregulated cAMP synthesis (Büssemaker *et al*., 2003). A more complete understanding of gender-dependent influences on gap junctional communication may ultimately provide new insights into the mechanisms that underlie patho-physiological changes in vascular reactivity. Oestrogens upregulate the expression of Cx43 and enhance EDHF-type responses in the rat mesenteric circulation (Liu *et al*., 2002), and in human pregnancy the relaxant response of myometrial arteries to ACh becomes entirely dependent on gap junctions, rather than NO (Kenny *et al*., 2002). By contrast, in cerebral arteries oestrogens inhibit EDHF-type relaxations, so that connexin-mimetic peptides attenuate the endothelium-dependent component of ADP-induced dilation in the pial circulation of ovariectomized rats, but not in controls (Golding & Kepler, 2001; Xu *et al*., 2002).

Finally, from a pharmacological perspective, evidence that electrotonic signalling may play a central role in vascular control should stimulate re-evaluation of the actions of many of the common pharmacological probes used to investigate the nature of the mechanisms regulating arterial tone. In addition to their more widely accepted actions, compounds as diverse as the Na^+/K^+-ATPase inhibitor ouabain, the CYP_{450} epoxygenase inhibitor clotrimazole, the cannabinoid receptor antagonist SR141716A and fenamate inhibitors of cyclo-oxygenase, which have all been employed experimentally to dissociate pathways contributing to the EDHF phenomenon, have now also been shown to exert major inhibitory effects on gap junctional communication (Chaytor *et al*., 1999; Harris *et al*., 2000; Harks *et al*., 2001; Brandes *et al*., 2002; Martin *et al*., 2003; Srinivas & Spray, 2003).

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