440P A PROGRAM FOR FITTING DATA FOR A SINGLE CURVE TO THE OPERATIONAL MODEL (BLACK EQUATION)

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441P EDHF EXISTS

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Endothelial cells synthesize and release vasoactive mediators in response to various neurohumoral substances (e.g. acetylcholine, adenosine triphosphate, bradykinin, thrombin, ...) and physical stimuli (e.g. shear stress exerted by the flowing blood). Nitric oxide (NO) produced by the L-arginine-NO synthase pathway and prostacyclin produced from arachidonic acid by the cyclooxygense pathway have been identified as endothelium-derived vasodilators. However, not all endothelium-dependent relaxations can be fully explained by the release of either NO or/and prostacyclin. Another unidentified substance(s) which hyperpolarizes the underlying vascular smooth muscle cells, termed endothelium-derived hyperpolarizing factor (EDHF), may contribute to endothelium dependent relaxations.

In blood vessels from various species, endothelium-dependent relaxations are partially or totally resistant to inhibitors of NO synthase and cyclooxygenase. In these blood vessels endothelium-dependent hyperpolarizations, which are resistant to NO scavengers and to inhibitors of NO synthase and cyclo-oxygenase, are also observed without an increase in intracellular level of cyclic nucleotides (cGMP and cAMP) in the smooth muscle. In canine, porcine and human blood vessels, the hyperpolarization cannot be mimicked by nitrovasodilators or exogeneous NO. However in other species (rat, guinea-pig, rabbit), endothelium-dependent hyperpolarizations resistant to NO synthase and cyclooxygenase and hyperpolarizations to endothelium-derived or exogeneous NO can be observed in the same vascular smooth muscle cells. In most blood vessels where NO causes hyperpolarization, the response is blocked by glibenclamide suggesting the involvement of ATP-dependent potassium channels. However, hyperpolarizations caused by EDHF are insensitive to glibenclamide but, depending on the tissue, are inhibited by relatively small concentration of TEA, or by apamin or the combination of charybdotoxin plus apamin, clearly indicating that NO and EDHF interacts with two different targets.

The existence of EDHF as a diffusable substance has been demonstrated under superfusion bioassay conditions whereby the source of EDHF was either native vascular segments or cultured endothelial cells, using either conventional intracellular microelectrode or patch-clamp techniques. Under the same bioassay conditions, EDHF released from cultured endothelial cells reduces the intracellular calcium concentration in vascular smooth muscle cells, supporting the involvement of this factor in the endothelium-dependent relaxation of the vascular smooth muscle cells. The technical difficulties in demonstrating the diffusable nature of EDHF could be explained either by a very short half-life of the substance, its preferential abluminal release, the simultaneous release of a hypothetical endothelium-derived depolarizing factor or a combination of these possibilities.

Theoretically, endothelium-dependent hyperpolarization may also involve electrical coupling through myo-endothelial junctions. Indeed, substances which produce endothelium-dependent hyperpolarization of vascular smooth muscle cells also hyperpolarize, with the same time course, endothelial cells. However, dye studies do not demonstrate coupling between endothelial and smooth muscle cells. Furthermore, although electrical coupling from smooth muscle to endothelial cells exists, electrical propagation in reverse direction does not seem to occur. Finally, halothane or heptanol, agents which uncouple cells linked by gap junctions, do not inhibit endothelium-dependent hyperpolarizations.

Altogether these results suggest the existence of a third pathway, besides the L-arginine-NO synthase and the cyclooxygenase pathways, in the endothelium production and release of vasoactive factors. The identification of EDHF and/or the discovery of specific inhibitors of its synthesis and its action may allow a better understanding of its physiological and pathophysiological role(s).

442P EDHF AND VASCULAR SMOOTH MUSCLE: ION CHANNEL ASPECTS

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Evidence that a hyperpolarizing factor (EDHF; see Taylor & Weston, 1988) could be liberated from the vascular endothelium was obtained more than 13 years ago (Bolton *et al.*, 1984). However, the significance of this observation went largely unrecognised until a series of membrane potential and ion flux experiments showed that nitric oxide (NO)-donors did not mimic the actions of EDHF (Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; Taylor *et al.*, 1988). Although it now seems likely that NO (EDRF) can hyperpolarize certain vascular muscles, evidence in favour of the separate existence of both EDHF and EDRF is overwhelming (Garland *et al.*, 1995).

Over the past few years, a profile has been built up of the K-channel opening actions of EDHF in the rat hepatic artery. In this vessel, EDHF can be antagonized by a mixture of apamin *plus* charybdotoxin, inhibitors of small and large conductance, Ca^{2+}-sensitive K-channels (SK_{Ca} and BK_{Ca}, respectively), whereas each toxin alone is ineffective. Since neither iberiotoxin nor kaliotoxin can substitute for charybdotoxin (Zygmunt & Högestätt, 1996), it is most unlikely that BK_{Ca} is the channel opened by EDHF.
Charybdotoxin inhibits not only BK_{Ca} but also delayed rectifier (K_V) channels comprising the gene products Kv1.2 and Kv1.3 (Kaczorowski *et al.*, 1996). Furthermore, the K_V inhibitor, ciclazindol partially antagonises the actions of EDHF in the hepatic artery and, in combination with charybdotoxin, abolishes the actions of this factor (Zygmunt *et al.*, 1997). On theoretical grounds, however, it seems unlikely that a voltage-sensitive channel such as K_V (or BK_{Ca}) could be responsible for the actions of EDHF. Under normal conditions, such a channel would be tightly closed at membrane potentials comparable to those achieved during EDHF-induced hyperpolarisations and other inhibitors of the delayed rectifier such as 4-aminopyridine, terikalant and dofetilide do not antagonize EDHF (Zygmunt & Högestätt, 1996: Zygmunt *et al.*, 1997).

These studies in rat hepatic artery collectively suggest that the K-channel opened by EDHF is structurally-related to K_V and BK_{Ca} and that it could be an apamin-insensitive variant of SK_{Ca} (see Köhler *et al.*, 1996). Such a channel is voltage-insensitive and would thus be able to sustain the membrane hyperpolarization which is one of the axiomatic properties of EDHF. The view that the target for EDHF is an SK_{Ca}-like member of K-channel Superfamily 1 would also hold for those vessels in which either charybdotoxin or apamin alone antagonises EDHF.

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443P ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR: IS THE ANSWER NO?

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Nitric oxide (NO), by stimulating the production of vascular smooth muscle cyclic GMP, is thought to account for endothelium-dependent vasodilation. Several other mediators have been proposed to fulfill the role of an endothelium-derived hyperpolarizing factor (EDHF) which hyperpolarizes and relaxes smooth muscle even in the presence of NO synthase inhibitors, and in the absence of a measurable rise in cyclic GMP. However, it is likely that NO itself possesses all the properties of, and can account for EDHF (Cohen *et al.*, 1996). In isolated segments of rabbit carotid artery, acetylcholine stimulates the release of NO and a consequent elevation of cyclic GMP, hyperpolarization and relaxation of the smooth muscle (Cowan *et al.*, 1993; Cohen *et al.*, 1997). The changes in membrane potential and force are resistant to NO synthase inhibitors (Cohen *et al.*, 1997; Plane *et al.*, 1996), but the rise in cyclic GMP is blocked (Cowan *et al.*, 1993), suggesting responses independent of the cyclic nucleotide. The relaxation in the presence of NO synthase inhibitors, but not that which occurs in their absence, is prevented by K^+ channel inhibition with charybdotoxin (Cowan *et al.*, 1993), suggesting GMP-independent hyperpolarization. The release of NO has been shown by chemical measurements to persist in the presence of NO synthase inhibitors, and the responses of the smooth muscle to correlate with NO release (Cohen *et al.*, 1997), rather than to the level of cyclic GMP (Cowan *et al.*, 1993). This indicates a cyclic GMP-independent mechanism by which NO can cause hyperpolarization and relaxation of smooth muscle. The later mechanism of hyperpolarization is supported by studies showing that, independently of cyclic GMP elevation, exogenous NO, or that released from endothelium in response to acetylcholine, can activate calcium-dependent potassium channels in isolated smooth muscle membrane patches (Bolotina *et al.*, 1994). Furthermore, relaxation to exogenous NO persists after blocking the rise in cyclic GMP and, like the residual response to acetylcholine, becomes sensitive to charybdotoxin (Bolotina *et al.*, 1994). These studies indicate that 1) NO is released in sufficient concentration from the endothelium to cause hyperpolarization and relaxation of the smooth muscle, 2) the response of smooth muscle membrane potential and relaxation both correlate with the concentration of NO released by the endothelium, 3) exogenous NO or that released from the endothelium can activate K^+ channels directly and hyperpolarize and relax smooth muscle by that mechanism, without reliance upon cyclic GMP, and 4) although the response of the smooth muscle to endothelium-derived NO is normally accompanied by an elevation of smooth muscle cyclic GMP, its actions on smooth muscle K^+ channels, membrane potential, and relaxation apparently do not strictly depend on the cyclic nucleotide. Thus, in addition to its role as a relaxing factor, NO possesses all the physiological and chemical characteristics of endothelium-derived hyperpolarizing factor in the rabbit carotid artery.

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444P IS EDHF A P450-DEPENDENT METABOLITE OF ARACHIDONIC ACID?

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The endothelium-derived hyperpolarizing factor (EDHF) is transiently released following the application of Ca^{2+}-elevating agonists, while it seems to be continuously produced in response to rhythmic vessel distension. In isolated coronary arteries under conditions of combined nitric oxide (NO) synthase (NOS)/ cyclo-oxygenase blockade, rhythmic oscillations in intraluminal pressure, which led to simultaneous changes in vessel diameter, elicited the release of EDHF. The magnitude of the EDHF-induced hyperpolarization, as assessed in a patch clamp bioassay system, was proportional to the amplitude of vessel wall distension. Although the physiological significance of EDHF has not yet been fully elucidated, our recent experimental evidence suggests a role for EDHF in the control of arterial compliance.

Pharmacological characterisation of EDHF derived from agonist-stimulated porcine coronary and rabbit carotid and mesenteric arteries implies that this factor is a P450-dependent metabolite of arachidonic acid, possibly an epoxyeicosatrienoic acid (EET). Indeed, the production of the agonist- and pulsatile stretch-induced release of EDHF in bioassay experiments can be inhibited by the suicide P450 inhibitor, 17-octadecynoic acid (17-ODYA). Although the exact identity of EDHF synthase remains to be elucidated experimental data suggest that it may be a cytochrome P450 2C (CYP 2C) isoform which synthesises vasodilatory, Ca^{2+}-dependent K^+ channel (K^+_{Ca})-activating EET's. Induction of P450 enzymes by β-naphthoflavone not only increases the expression of CYP 2C, it also enhances the production of EDHF from cultured and native endothelial cells.

Recently, a specific cannabinoid receptor (CB1) antagonist, SR141716A, has been shown to attenuate EDHF-induced reiaxation of isolated rat mesenteric vessels while anandamide (arachidonoylethanolamide), an endogenous ligand for central cannabinoid receptors elicited "EDHF-like" relaxations. However, although anandamide and the CB1 agonist, HU 210, induced relaxation of mesenteric vessels by a cyclooxygenase-dependent mechanism, these compounds did not induce the EDHF-like (N^Gnitro-L-arginine/diclofenac- insensitive) dilation of either porcine coronary arteries or rabbit carotid and mesenteric arteries. Moreover SR141716A failed to inhibit EDHF-mediated dilations in any of the preparations studied.

Since a maximal EDHF response can only be observed in the presence of N^Gnitro-L-arginine and diclofenac, it appears that the production of EDHF, may be affected by physiological concentrations of NO and prostacyclin. Indeed, under conditions of combined NOS/cyclo-oxygenase blockade NO donors are able to attenuate the EDHF-mediated relaxation of arterial rings. Using a patch clamp bioassay for EDHF we have been able to demonstrate that NO attenuated the production of EDHF rather than interfering with its ability to activate K^+_{Ca} channels in detector vascular smooth muscle cells. Physiological concentrations of NO, either generated by NOS III or NOS II are able to attenuate P450 activity acutely, via a mechanism which may involve a decrease in $[Ca^{2+}]_i$, and chronically, by decreasing the expression of P450 protein. Proinflammatory cytokines, which induce the expression of NOS II in porcine aortic endothelial cells, and the prolonged incubation of endothelial cells with NO donors decrease the expression of CYP 2C and attenuate EDHF-mediated relaxations suggesting that these two endothelial autacoids interact with each other at the level of expression and activity.

Taken together our results suggest that a P450 inhibitor sensitive, hyperpolarizing autacoid can be released from the endothelium in response to mechanical and agonist stimulation. Although the synthesis of this autacoid is attenuated by NO in conduit arteries, EDHF seems to play a major role in vessels which produce less NO, such as the resistance-sized arteries within the coronary and mesenteric circulations.

445P EDHF: IS THE ANSWER A CANNABINOID?

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The identity of EDHF has been a matter of controversy for sometime, with candidates including epoxides of arachidonic acid. However, we have recently proposed that an EDHF may be one of the recently identified endogenous cannabinoids (Randall *et al.*, 1996). This proposal was based on the observation that, in the rat mesenteric and coronary vasculatures, EDHF-mediated relaxations were selectively opposed by the cannabinoid receptor antagonist, SR141716A (1μM). Furthermore, in conscious rats treatment with SR141716A (10mg kg^{-1} i.v.), in the presence of NO synthase blockade, also opposed the depressor and regional vasodilator effects of bradykinin. Coupled to these observations, anandamide, the prototype endocannabinoid derived from arachidonic acid and ethanolamine, caused potent vasorelaxations in the mesenteric (Randall *et al.*, 1996) and coronary (Randall & Kendall, 1997) vasculatures, which were endothelium-independent. The vasorelaxant responses to anandamide in the mesentery were entirely blocked by raising extracellular K^+ (60mM) and sensitive to 10mM tetraethylammonium but unaffected by 10μM glibenclamide, whilst those in the heart were sensitive to 300nM tetrabutylammonium. These results, therefore, point to anandamide causing vasorelaxation through hyperpolarization or repolarization due to K^+ channel activation. In the mesentery the vasorelaxant effects of anandamide are not mimicked by exogenous anandamide, whilst HU210, a stable cannabinoid agonist, also causes vasorelaxation which is sensitive to SR141716A and 60mM K^+.

The involvement of epoxides of arachidonic acid, derived via a cytochrome P450 monooxygenase, in EDHF-mediated responses is partly suggested by the ability of cytochrome P450 inhibitors to inhibit these responses. However, recent evidence has suggested that these agents may act as EDHF inhibitors by causing K^+-channel blockade. In the mesentery the cytochrome P450 inhibitors proadifen (10μM) and clotrimazole (10μM, also in the heart) inhibited both EDHF-mediated responses and those induced by anandamide, thereby drawing further parallels between EDHF and endocannabinoids (Randall *et al.*, 1997).

In mesenteries prelabelled with tritiated arachidonic acid, conditions which evoke EDHF release were associated with the release of an arachidonic acid metabolite, which on extraction and thin layer chromatography co-migrated with authentic anandamide (Randall *et al.*, 1996). In more recent preliminary experiments, prelabelling with tritiated ethanolamine resulted in the appearance of acylethanolamides on stimulation of EDHF release. These findings give preliminary chemical evidence that an EDHF may be an endocannabinoid.

The above findings give clear support to the hypothesis that an endocannabinoid may be an EDHF. However, much work is required to characterise fully the vascular actions of the endocannabinoids and to confirm the chemical identity of endocannabinoids as EDHFs.

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Endothelium-dependent hyperpolarization of vascular smooth muscle contributes to the vasodilatation evoked by endothelial stimulants. Since the rectifying properties of myo-endothelial gap junctions impede the electrotonic transfer of hyperpolarization from endothelial cells to vascular smooth muscle cells, it is often assumed that a diffusable chemical is involved. Besides NO and prostanoids, endothelial cells produce several compounds capable of inducing hyperpolarization of vascular smooth muscle cell plasma membrane. Thus, during inhibition of the synthesis of both NO and prostaglandins, a component of the endothelium-dependent relaxation to agonists is induced by hyperpolarization and has been attributed to an endothelium-derived hyperpolarizing factor(s) (EDHF).

Carbon monoxide (CO) has been proposed as an EDHF in porcine pulmonary artery based on the inhibition of the relaxation attributed to EDHF by Tin protoporphyrin IX, a heme oxygenase inhibitor. Although the presence of heme oxygenases in endothelial cells is consistent with this idea, the relaxation presumably mediated by EDHF is resistant to oxyhemoglobin (a scavenger of both NO and CO) in canine arteries. H_2O_2 induces hyperpolarization in vascular smooth muscle in porcine coronary artery. However, EDHF-mediated responses are not affected by catalase, a scavenger of H_2O_2 in both porcine and canine coronary arteries.

Anandamide, an endocannabinoid, evokes relaxation in isolated rat mesenteric artery. In this artery, the relaxation attributed to EDHF is impaired by the CB-1 receptor antagonist SR141716A [N-(piperidin-1-yl)-5-(-4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], the selectivity of which warrants further investigation.

Epoxyeicosatrienoic acids (EETs) induce vascular smooth relaxation by activating large conductance Ca^{2+}-activated K^+ channels. EETs are generated by endothelial cells, through an arachidonic acid epoxygenase pathway(s). Accordingly, in several arteries, cytochrome P450 inhibitors suppress both the release of EETs and endothelium-dependent hyperpolarization and relaxation of vascular smooth muscle attributed to EDHF.

K^+ channels are the targets of EDHF. The involvement of different classes of K^+ channels (e.g. small and large-conductance Ca^{2+}-activated channels) suggests either the existence of several EDHFs, or that EDHF requires a promiscuis transducing mechanism that enables it to interact with different effectors. Thus, the nature of the relationship between EDHF and K^+ channels needs to be assessed further.

Unresolved issues include the lack of specific pharmacological tools to address the physiological roles of the aforementioned candidates as EDHFs. Different K^+ channel blockers affect EDHF-mediated responses depending on species and anatomical origin of the vessel. It is premature to dismiss any of the candidates, since their relationships with K^+ channels are not fully described. In some arteries cytochrome P450 inhibitors are ineffective, in contrast to phospholipase A_2 inhibitors. In canine arteries, this is due to pre-formed EETs that are mobilized by phospholipases. Hence, resistance to P450 inhibitors may not be a sufficient criterion to rule out EETs as EDHF. EDHF has been difficult to detect using cascade bioassays. The poor hydrosolubility of these lipid derivatives could account for this. Indeed, albumin faciliates detection of EDHF using cascade bioassays. However, more studies are needed to assess the compatibility of the metabolism and chemistry of putative candidates with those attributed to EDHF.

447P CERAMIDE/STRESS KINASE-MEDIATED APOPTOSIS

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The endotoxic shock syndrome is characterized by systemic inflammation, multiple organ damage, circulatory collapse and death. Excessive systemic release of tumor necrosis factor (TNF)α and other cytokines purportedly mediates this process. However, the primary tissue target remains unidentified.

The present studies provide evidence that endotoxic shock results from disseminated endothelial apoptosis. Injection of lipopolysaccharide (LPS), and its putative effector TNFα, into C_{57}BL/6 mice induced apoptosis in endothelium of intestine, lung, fat and thymus after 6 hours, preceding non-endothelial tissue damage. LPS or TNFα injection was followed within one hour by tissue generation of the pro-apoptotic lipid, ceramide. TNF-binding protein, which protects against LPS-induced death, blocked LPS-induced ceramide generation and endothelial apoptosis, suggesting that systemic TNF is required for both responses. Acid sphingomyelinase knockout mice displayed defects in LPS-induced endothelial apoptosis and animal death, defining a role for ceramide in mediating the endotoxic response. Further, intravenous injection of basic fibroblast growth factor, which acts as an intravascular survival factor for endothelial cells, protected mice against LPS-induced endothelial apoptosis and animal death.

These investigations demonstrate that LPS induces a disseminated form of endothelial apoptosis, mediated sequentially by TNF and ceramide generation, and suggest that this cascade is mandatory for evolution of the endotoxic syndrome.