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Role of Gap Junctions in the Endothelium-Dependent Hyperpolarization of Vascular Smooth Muscle Cells

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Hyperpolarization of arterial smooth muscle by acetylcholine is considered to be produced by the release of an unidentified chemical substance, an endothelium-derived hyperpolarizing factor (EDHF). Several chemicals have been proposed as the candidate for EDHF. However, none of them fulfil completely the nature and property of EDHF. Ultrastructural observation with electron microscope reveals that in some arteries, gap junctions are formed between endothelial and smooth muscle cells. In small arterioles, injection of gap junction permeable dyes into an endothelial cell results in a distribution of the dye to surrounding cells including smooth muscle cells. These observations allow the speculation that myoendothelial gap junctions may have a functional significance. Simultaneous measurement of the electrical responses in both endothelial and smooth muscle cells using the double patch clamp method demonstrates that these two cell types are indeed electrically coupled, indicating that they behave as a functional syncytium. The EDHF-induced hyperpolarization is produced by an activation of Ca^{2+}-sensitive K^+-channels that are inhibited by charybdotoxin and apamin. Agonists that release EDHF increase $[Ca^{2+}]_i$ in endothelial cells but not in smooth muscle cells. Inhibition of gap junctions with chemical agents abolishes the agonist-induced hyperpolarization in smooth muscle cells but not in endothelial cells. All these observations can be explained if EDHF is an electrotonic signal propagating from endothelium to smooth muscle cells through gap junctions.

Key Words: EDHF, Gap junction, Hyperpolarization, Potassium channel, Calcium

INTRODUCTION

Vascular endothelial cells release an endothelium-derived relaxing factor (EDRF) in response to chemical and physical stimuli, and this factor relaxes vascular smooth muscles (Furchgott, 1984; Vanhoutte et al, 1986). The actions of EDRF resemble those of nitro-containing compounds, which led to the discovery that EDRF is nitric oxide (NO) produced from L-arginine (Ignarro et al, 1987; Palmer et al, 1987). NO produced in endothelial cells diffuses into smooth muscle cells and stimulates guanylate cyclases to elevate cytosolic production of cyclic GMP, which results in an reduction of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through the activation of Ca^{2+}-pumps. This decrease in $[Ca^{2+}]_i$ induces muscle relaxation (Moncada et al, 1991). Most blood vessels receive only an adrenergic innervation, and noradrenaline released from these nerves produces muscle constriction through the activation of α-adrenoceptors (Furness & Costa, 1987). The dilatation of blood vessels is, therefore, mediated by non-neural component, EDRF. Thus, the local regulation of the caliber of many arteries arises from the competition between neuronal constrictor and endothelial dilator mechanisms.

In many vascular tissues, acetylcholine (ACh) hyper-

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polarizes the smooth muscle membrane, in the absence of a cholinergic innervation (Bolton & Large, 1986; Suzuki, 1989). As this hyperpolarization disappears after the removal of the endothelial cells, the potential is originally considered to be produced by EDRF (Bolton et al, 1984). In the smooth muscle of the rabbit saphenous artery, ACh also hyperpolarizes the membrane and produces a relaxation in an endothelium-dependent manner. However in this artery, oxotremorine, a muscarinic agonist, produces relaxation without membrane hyperpolarization (Komori & Suzuki, 1987a). Antagonism of the ACh-induced hyperpolarization and relaxation by muscarinic antagonists differed in their potency, indicating that ACh released EDRF and EDHF through different subtypes of muscarinic receptor (Komori & Suzuki, 1987b). In rat blood vessels, the ACh-induced hyperpolarization remains unaltered after inhibiting the actions of EDRF directly with oxyhemoglobin, or indirectly upon blockade of guanylate cyclase with methylane blue. These observations indicated that the ACh-induced hyperpolarization was produced by mechanisms different from the actions of EDRF. The unidentified substance that produced hyperpolarization of smooth muscle was termed the endothelium-derived hyperpolarizing factor (EDHF) (Chen et al, 1988). Endothelial cells are large sources of the production of prostanoids (Moncada & Vane, 1979), and the released prostacyclin also is able to dilate and hyperpolarize vascular smooth muscles (Parkington et al, 1995; Yajima et al, 1999). Thus, there are at least three endothelial factors that produce vasodilatation: EDRF, EDHF and prostacyclin. In this review, we would like to address the recent advances in our understanding of the role of gap junctions in the endothelium-dependent hyperpolarization in some blood vessels.

GENERAL PROPERTIES OF EDHF

Since the first proposal of the existence of EDHF (Chen et al, 1988), there has been a rapid accumulation of data related to the properties of EDHF, and they have been recently reviewed (Taylor & Weston, 1988; Komori & Vanhoutte, 1990; Suzuki & Chen, 1990; Suzuki et al, 1992; Garland et al, 1996). The general properties of EDHF could be summarized as follows.

EDHF hyperpolarizes vascular smooth muscles by activation of K^+-channels

In canine coronary artery, ouabain inhibits the ACh-induced hyperpolarization of the smooth muscle membrane, which suggests a contribution of electrogenic Na-K pump in the generation of these potential changes (Feletou & Vanhoutte, 1988). Partial contribution of Na-K pump in the EDHF-induced hyperpolarization is also observed in the rat mesenteric artery (Edwards et al, 1998). However, ouabain did not inhibit the ACh-induced hyperpolarization while it did block the hyperpolarization produced by activation of Na-K pump during withdrawal of K^+-free solution (Chen et al, 1989; Suzuki, 1989). An involvement of Na-K pump in the ACh-induced hyperpolarization is also not evident in other arteries (Chen et al, 1992; Fujii et al, 1992; Vanheel et al, 1994; Fukao et al, 1997). Thus, EDHF may not stimulate Na-K pump in vascular smooth muscle. Alternatively, the ACh-induced hyperpolarization observed in the canine coronary artery (Feletou & Vanhoutte, 1988) may be produced by substances other than EDHF.

In rat arteries, ACh increases an efflux of incorporated Rb^+ in an endothelium-dependent manner (Chen et al, 1988). Moreover, the amplitude of the ACh-induced hyperpolarization decreases as the concentration of K^+ is raised (Bolton et al, 1984; Chen & Suzuki, 1989). These results indicate that the hyperpolarization is produced mainly by an increase in membrane conductance for K^+ (Suzuki & Chen, 1990). There are many types of K^+-channels in arterial smooth muscle (Nelson & Quayle, 1995) and endothelial membrane that may be responsible (Adams, 1994). The K^+-channels involved in the EDHF-induced hyperpolarization are considered to be glibenclamide-sensitive ATP-sensitive K^+-channels (Standen et al, 1989) or Ba^{2+}-sensitive inward rectifier K^+-channels (Edwards et al, 1998). This has not been confirmed in other arteries. The ACh-induced hyperpolarization is inhibited by CTX and apamin in the submucosal arterioles of the guinea-pig (Hashitani & Suzuki, 1996), the rat hepatic artery (Zygmunt & Hgesttt, 1996), the guinea-pig coronary artery (Nishiyama et al, 1998) and the rabbit mesenteric artery (Murphy & Brayden, 1995). These results indicate that the K^+-channels involved are mainly Ca^{2+}-activated K^+-channels.

EDHF is involved in the endothelium-dependent relaxation

In rat arteries, ACh produces an endothelium-dependent relaxation, after inhibition of the production of NO and prostanoids (Chen et al, 1988; Chen & Suzuki, 1989; Suzuki & Chen, 1990). This residual relaxation is considered to be produced by EDHF, and is termed an EDHF-attributed relaxation, since it disappears in high-K solution where EDHF cannot hyperpolarize the membrane. The EDHF-attributed relaxation is inhibited by CTX and apamin (Zygmunt & Hgesttt, 1996), suggesting that the response is produced by an increased activation of Ca^{2+}-sensitive K^{+}-channels.

EDHF is the major relaxant in peripheral blood vessels

Comparison of the EDHF-attributed relaxation with the EDRF-induced relaxation in smooth muscles obtained from different regions of vascular beds indicates that the ratio of the EDHF-attributed component is small in large arteries and it increases in peripheral arteries (Nagao et al, 1992; Garland et al, 1995; Zygmunt et al, 1995). That is, contribution of EDHF in the relaxation of arterial smooth muscle is more important than EDRF in peripheral arteries. On the other hand, EDRF is the major contributor of the endothelium-dependent relaxation in the proximal or large arteries.

CHEMICAL NATURE OF EDHF

Cascade experiments confirmed that EDRF was a diffusible substance (Furchgott & Zawadzki, 1980). Attempts have been made to confirm that EDHF is also a humoral substance in several arteries, and the results are equivocal. In rat arteries, ACh hyperpolarizes the membrane of endothelium-denuded arterial muscles only when the endothelium-intact artery is located upstream to the system (Kauser et al, 1989), suggesting that endothelial cells may release a diffusible substance that hyperpolarizes arterial smooth muscle. The sandwich preparations consisting of intact and endothelium-removed coronary arteries of the guinea-pig reveal that ACh can release diffusible hyperpolarizing factor from the endothelial cells (Chen et al, 1991). On the other hand, in the porcine coronary artery, no EDHF-attributed relaxation is elicited in the recipient artery by stimulation of the donor artery with ACh (Kauser & Rubanyi, 1992) or bradykinin (Hecker et al, 1994). In the same artery, however, bradykinin releases humoral substance that activates Ca^{2+}-dependent K^{+}-channels and hyperpolarizes cultured aortic smooth muscle cells (Popp et al, 1996).

Several chemicals are proposed as the candidate for EDHF, and they include: metabolites of arachidonic acid produced through activation of the enzyme cytochrome P450 (Hecker et al, 1994; Campbell et al, 1996; Popp et al, 1996), cannabinoids produced from arachidonic acid (Randall et al, 1996), K^{+} released from endothelial cells (Edwards et al, 1998), or H_2O_2 derived through activation of endothelial nitric oxide synthase (Matoba et al, 2000). However at this moment, none of these candidates seem to fulfil the properties of endothelium-derived hyperpolarizing factor.

MYOENDOTHELIAL GAP JUNCTIONS

Ultrastructural observations using the electron microscope reveal that in many vascular tissues, endothelial and smooth muscle cells are connected by gap junctions (Spagnoli et al, 1982; Bény & Connat, 1992; Sandow & Hill, 2000). Functional communications between these two types of cells are also suggested by the diffusion of dyes injected into endothelial cells to surrounding cells including smooth muscle cells, presumably through gap junctions (Bény, 1990, 1997; Little et al, 1995). In arterioles of the hamster, a large molecular dye injected to an endothelial cell diffuses into surrounding endothelial cells alone, whereas a dye of small molecules diffuses to both endothelial and smooth muscle cells (Segal & Bény, 1992; Little et al, 1995). In the porcine coronary artery, membrane potential changes of smooth muscle cells produced upon stimulation of β-adrenoceptors (Bény & Pacicca, 1994), tetrabutylammonium (von der Weid & Bény, 1993) or current injection through an intracellular electrode (Bény, 1997) could also be recorded in the endothelial cells. In mesenteric arterioles of the guinea-pig, high concentrations (5～10 mM) of Ba^{2+} depolarize the membrane and elicit spike potentials in both endothelial and smooth muscle cells (Yamamoto et al, 1998). As voltage-dependent ion channels are distributed in smooth muscle cells but not in endothelial

cells (Adams, 1994; Nelson & Quayle, 1995), the spike potentials recorded in the endothelial cells must be propagated from the smooth muscle cells in an electrotonic manner. These electrophysiological experiments indicate that there is a functional communication between endothelial and smooth muscle cells, such that electrical signals appearing in smooth muscle cells can propagate to surrounding cells including endothelial cells through gap junctions.

In cultured bovine aortic endothelial cells, ACh hyperpolarizes the membrane and elevates $[Ca^{2+}]_i$ (Busse et al, 1988; Schlling, 1989; Sakai, 1990). Elevation of $[Ca^{2+}]_i$ by ACh is also shown in intact endothelial cells of the submucosal arterioles of guinea-pig small intestine (Fukuta et al, 1999a). Interestingly, this increase in endothelial $[Ca^{2+}]_i$ was not accompanied by any elevation of $[Ca^{2+}]_i$ in the vascular smooth muscle cells. That is, ACh elevates endothelial $[Ca^{2+}]_i$ but not smooth muscle $[Ca^{2+}]_i$. ACh hyperpolarizes the endothelial cell membrane in the rat mesenteric artery (Chen & Cheung, 1992), the rat aorta (Marchenko & Sage, 1996) and the rabbit pulmonary valve (Ohashi et al, 1998), and these potentials are inhibited by charybdotoxin (CTX) and apamin, known inhibitors of Ca^{2+}-sensitive K^+-channels (Nelson & Quayle, 1995). The endothelium-dependent hyperpolarization of arterial smooth muscle cells is also inhibited by CTX and apamin in the rabbit mesenteric artery (Murphy & Brayden, 1995), the submucosal arterioles of guinea-pig small intestine (Hashitani & Suzuki, 1996) and the coronary artery of guinea-pig (Nishiyama et al, 1998). In these cases, it is reasonable to speculate that the endothelium-dependent hyperpolarization of smooth muscle cells produced by ACh is an electrotonic potential propagated from endothelial cells through gap junctions, in an electrotonic manner. This speculation may be proved experimentally by demonstrating that the inhibition of ACh-induced hyperpolarization occurs upon blockade of gap junctional communication.

GAP JUNCTION INHIBITORS

Heptanol, octanol or halothane is known as an inhibitor of gap junctions in cardiac muscles (Spray & Burt, 1990). In porcine coronary artery, heptanol inhibits electrical communications between endothelial and smooth muscle cells (Bny & Pacicca, 1994). Diffusion of lucifer yellow from endothelial cells to smooth muscle cells is also inhibited by heptanol in arterioles of the hamster cheek pouch (Little et al, 1995). However, these gap junction inhibitors do not effectively block electrical communications between endothelial and smooth muscle cells in submucosal arterioles of the guinea-pig small intestine (Hashitani & Suzuki, 1997; Yamamoto et al, 1998).

Glycyrrhetinic acid, a kind of saponin extracted from the root of licorice, blocks gap junctional communications in human fibroblast cell lines (Davidson et al, 1986) and liver epithelial cells (Guan et al, 1996). The selective inhibition by an isoform of glycyrrhetinic acid (18 α-glycyrrhetinic acid) of the EDHF-attributed relaxation is reported in the rat aorta (Taylor et al, 1998). Using double electrode whole-cell patch clamp methods, 18 β-glycyrrhetinic acid is shown to inhibit selectively the gap junctional communications between endothelial and smooth muscle cells of the mesenteric arterioles of the guinea-pig (Yamamoto et al, 1998; 1999). The EDHF-attributed relaxation produced by ACh is also inhibited by 18-β glycyrrhetinic acid (Fukuta et al, 1999b; Imaeda et al, 2000). Carbenoxolone, a water soluble form of 18 β-glycyrrhetinic acid, is also effective in inhibiting the ACh-induced hyperpolarization and EDHF-attributed relaxation in the rat hepatic artery (Edwards et al, 1999; 2000) and the guinea-pig aorta (Kamei et al, 2000).

MODULATION OF ACH-INDUCED HYPERPOLARIZATION BY GAP JUNCTION INHIBITOR

In mesenteric arterioles of the guinea-pig, ACh hyperpolarizes the membrane of endothelial cells with two phases, an initial fast component followed by a slow sustained component. Hyperpolarizations with a similar form and time course are also evoked by ACh in smooth muscle cells of these arterioles. Simultaneous measurement of membrane potential from these two types of cells reveals that the amplitude of hyperpolarization is smaller in smooth muscle cell than in endothelial cell by a few percent (Y. Yamamoto, unpublished observation). This suggests a decremental propagation of endothelial hyperpolarization to smooth muscle cells.

The endothelial cell membrane is still hyperpolarized by ACh after blocking intercellular gap junctional electrical communications with 18 β-glycyrr-

hetinic acid (Yamamoto et al, 1999). Blockade of intercellular gap junctional electrical communications indicates that the recording cell is functionally isolated from the surrounding cells, and this agrees with the evidence that ACh hyperpolarizes enzymatically isolated endothelial cells (Busse et al, 1988; Sakai, 1990). The endothelial hyperpolarization is produced by an activation of Ca^{2+}-sensitive K^+-channels (Olesen et al, 1988; Chen & Cheung, 1992; Yamamoto et al, 1999). The endothelial Ca^{2+} responses elicited by ACh are biphasic. The initial transient and following sustained components may be produced by Ca^{2+} released from internal stores through increased production of inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} entered from the external media through nonselective cation channels, respectively (Chen & Cheung, 1992; Schlling & Elliott, 1992).

In smooth muscle cells of the guinea-pig mesenteric arterioles, ACh also hyperpolarizes the membrane with time courses similar to those seen in endothelial cells. However, the muscular hyperpolarization is inhibited by blocking gap junctional communication with 18 β-glycyrrhetinic acid (Yamamoto et al, 1999). If EDHF is a humoral factor and stimulates smooth muscle cells from the outside of the membrane, blockade of gap junctional communications should not alter the responses of the smooth muscle cells. That is, the disappearance of ACh-induced hyperpolarization in smooth muscle cells after blockade of gap junctional communications could be explained by assuming that EDHF is not a humoral substance, but is an electrotonic potential propagated from endothelial cells through gap junctions.

In submucosal arterioles, ACh stimulates muscarinic receptors on the membrane and elevates intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in the endothelial cells but not smooth muscle cells (Fukuta et al, 1999a). It is therefore reasonable to speculate that the Ca^{2+}-sensitive K^+-channels activated may be distributed on the endothelial membrane but not on smooth muscle membranes. A direct electrical signaling from endothelial cells to smooth muscle cells through gap junctions has been suggested in some arterioles (Daut et al, 1988), and the mesenteric arterioles of the guinea-pig (Yamamot et al, 1999) may be the first demonstration of this signal transduction. These relationships between endothelial and smooth muscle cells may be reasonable for small arterioles, since there is a single muscle layer and it would be easy to regulate membrane potential of smooth muscle cells directly. Alternatively, it is also possible to speculate that endothelial substances such as IP_3 and Ca^{2+} are transported to the smooth muscle cells through gap junctions to stimulate the muscle membrane directly from the inside, since gap junctions have a large pore at the center through which substances with small diameters may be permeable (Spray & Burt, 1990). In this case, EDHF may be an intercellular messenger and not detectable as a paracrinic substance. In cascade experiments, the putative EDHF seems to act from the outside, since the perfusates of the intact porcine aorta activate Ca^{2+}-dependent K^+-channels and hyperpolarize the membrane of cultured aortic smooth muscle cells (Popp et al, 1996). This suggests that the putative EDHF, if any, may not be a gap junction permeable substance.

In considering large arteries with many layers of smooth muscle cells, electrical signals arising from endothelial cells may not propagate far and therefore may be less effective at modulating the membrane potential of cells at the outer edges of the vessel wall. Analysis of the ACh-induced relaxation in vessels isolated from various regions of some laboratory animals indicate that the ratio of the EDHF-attributed relaxation increases in peripheral arteries compared to the proximal elastic arteries whereas the opposite is the case for the NO-mediated relaxation (Nagao et al, 1992; Garland et al, 1995; Zygmunt et al, 1995). These observations suggest that changes in membrane potential are not the main factor in regulating vessel diameter in large arteries, rather a regulation of the pharmaco-mechanical coupling is taking a more important role.

CONCLUSION

EDHF was considered initially a humoral substance derived from the endothelial cells. However, careful experiments indicate that the agonist-induced hyperpolarization appears first in endothelial cells and is transferred to smooth muscle cells. In arterial tissues, there are myoendothelial gap junctions and they may be an important pathway to conduct electrical signals between smooth muscle and endothelial cells. These results suggest that in vascular beds EDHF is an electrical signal conducted from endothelial cells through gap junctions.

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