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Endothelium is required in the vascular spasm induced by tetraethylammonium and endothelin-1 in guinea-pig aorta

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1 To investigate the role of endothelium in vascular spasm, we studied the influence of endothelin-1 (ET-1) on the contracting and spasmogenic effect of the K^+-channel blocker, tetraethylammonium (TEA), in aorta rings of reserpine-treated guinea-pigs, perfused with either control (5.5 mM) or elevated (50 mM) glucose concentration.

2 Endothelium-dependent relaxation induced by acetylcholine was lost in rings contracted by noradrenaline in the presence of elevated glucose. In control medium, TEA (1–20 mM) induced a sustained tonic contraction, followed by a phasic spasm, characterized by rhythmic contractions. Elevated glucose, ET-1 (3 nM), or both, reduced the EC_{50} of TEA-induced tonic contraction, without modifying the maximum contractile effect.

3 In control medium, ET-1 reduced the time before TEA-induced spasm and increased the rate of rhythmic contractions. TEA-induced spasm was abolished by elevated glucose, and restored by ET-1. The spasm induced by TEA and ET-1 was amplified by the ET_A antagonist, EMD_{94246}, and suppressed by the ET_A-ET_B antagonist, bosentan. In endothelium-denuded vessels incubated with high glucose and ET-1, TEA evoked only a tonic contraction.

4 In control medium, L-NAME (N^G-nitro-L-arginine methyl ester) abolished TEA-induced rhythmic contractions. L-arginine, but not D-arginine, prevented the effect of L-NAME. In the presence of elevated glucose and ET-1, TEA-induced spasm was not affected by L-NAME, whereas verapamil, indomethacin, metyrapone, glybenclamide or apamin abolished the phasic spasm, unmasking the tonic contracture.

5 In conclusion, endothelium plays a regulatory role in the genesis and maintenance of TEA-induced rhythmic contractions, through the release endothelium derived relaxing factor and vasodilating eicosanoids.

Keywords: Endothelin; endothelium; glucose; K^+-channels; vasodilatation

Abbreviations: Ach, acetylcholine; EDRF, endothelium derived relaxing factor; ET-1, endothelin-1; GLU, glucose; Ind, indomethacin; K^+_{ATP} channels, ATP-sensitive K^+-channels; K^+_{Ca} channels, Ca^{2+}-activated K^+-channels; L-NAME, N^G-nitro-L-arginine methyl ester; NE, noradrenaline; PG_S, prostanoids; TEA, tetraethylammonium; Ver, verapamil

Introduction

The mechanisms controlling vascular tone are complex and appear largely impaired in different pathological conditions. Hypercholesterolemia (Jayakody *et al*., 1985), atherosclerosis (Freiman *et al*., 1986), ischaemia and reperfusion (Ku, 1982), acute and chronic hypertension (Lamping & Dole, 1987; Panza *et al*., 1990), congestive heart failure (Kubo *et al*., 1991), and diabetes (Oyama *et al*., 1986) are associated with abnormal endothelium-dependent vascular relaxation and increased vascular reactivity to different vasoactive substances, leading to vasospasm and tissue ischaemia. However, the mechanism triggering vascular spasm is currently unclear, although different factors or events seem to be involved. Threshold concentrations of the potent endogenous vasoconstrictor, endothelin-1 (ET-1), comparable to physiological plasma levels (Lerman *et al*., 1990), may play a role in the development of vascular spasm through a direct spasm induction and a sensitization of smooth muscle to various contracting agents (Aitekenead *et al*., 1990; La *et al*., 1990). Hypersensitivity of vascular smooth muscle, a cause of arterial spasm *in vivo*, may also be induced by a decrease in K^+ conductance, which leads to increased Ca^{2+} uptake and availability (Inoue *et al*., 1989; Wilde & Lee, 1989).

In order to investigate the mechanisms involved in vascular spasm, we studied the influence of threshold concentrations of ET-1 on the contracting and spasmogenic actions of tetraethylammonium (TEA), a blocker of large conductance Ca^{2+}-activated K^+ (K^+_{Ca}) channels (Benham *et al*., 1985; Uchida, 1985), in guinea-pig aorta rings. Vascular preparations were perfused either with a control medium, containing 5.5 mM glucose, or with elevated glucose concentration (50 mM), in order to mimic the impairment of endothelium-dependent relaxation observed *in vivo* in diabetic patients (Valentovic & Lubay, 1985; Saenz de Tajada *et al*., 1989; Tesfamariam *et al*., 1990; Calver *et al*., 1992; McVeigh *et al*., 1992; Hsueh & Anderson, 1992; Bohlen & Lash, 1993; Weisbrod *et al*., 1993; Dorigo *et al*., 1997). In fact, hyperglycaemia *per se* seems to be an independent risk factor for impaired endothelial cell function (Tesfamariam, 1994; Kuusisto *et al*., 1995) and a stimulus to increased secretion of ET-1 (Yamauchi *et al*., 1990).

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Methods

Dunkin-Hartley guinea-pigs of either sex (300–350 g) were treated with reserpine (2 mg kg^{-1}, i.p.) 48 and 24 h before sacrifice, in order to prevent the influence of noradrenaline, which might be released from sympathetic nerve terminals during the experiment (Temma *et al.*, 1977). The animals were then killed by cervical dislocation and exsanguinated. The thoracic aorta was removed and placed in a physiological salt solution of the following composition (mM): NaCl 120, KCl 2.7, $MgCl_2$ 0.9, NaH_2PO_4 0.4, $CaCl_2$ 1.37, $NaHCO_3$ 11.9 and glucose 5.5. The solution was bubbled vigorously with a mixture of 95% O_2 and 5% CO_2 which produced a pH of 7.5. The aorta was dissected free from connective tissue and cut into rings that were mounted vertically by means of stainless steel hooks in 10 ml organ baths containing the physiological solution, aerated as described above and maintained at 37.0 ± 0.3°C.

Changes in force were recorded isometrically by means of an E.C.T.A. isotonic transducer Linearcorder (Mark III, Watanabe, Japan). An initial load of 0.8 g was applied to the rings, which were then allowed to equilibrate for 2 h. Contractions were evoked by 1 μM noradrenaline (5 min contact time) at 30 min intervals, until three responses of equal amplitude were obtained (these responses corresponded to 80–90% of the maximal contractions induced by 120 mM KCl).

The relaxing response to aceylcholine (10 nM – 10 μM) was tested on the contraction induced by the last addition of noradrenaline and was considered as an index of endothelial functional integrity (Furchgott, 1983). The reactivity to acetylcholine in the presence of 5.5 mM glucose was stable over time, as previously demonstrated (Dorigo *et al.*, 1997). In experiments with denuded aorta rings, in which the endothelium had been removed by gently rubbing the intimal surface with a polyether string, the vessel preparations did not respond to acetylcholine.

Contractile responses to 1 μM noradrenaline and subsequent relaxing responses to 0.1 mM sodium nitroprusside were also compared in preparations with and without endothelium, in order to ensure that the smooth muscle was not damaged during the removal of endothelium. The mean force generated by noradrenaline in preparations with and without endothelium was 5.76 ± 0.09 and 5.95 ± 0.25 g, respectively ($n = 9$).

Sodium nitroprusside reduced the contractile effect of 1 μM noradrenaline by 93.20 ± 2.18% in preparations with endothelium, and by 95.31 ± 3.22% in the absence of endothelium ($n = 9$).

Where indicated, aortic rings were incubated with 50 mM glucose for 6 h. Lesser concentrations of glucose (44 mM) did not impair vascular reactivity to acetylcholine in a reproducible manner. Mannose (50 mM) was used as a hyperosmotic control. After 6 h, the arteries were contracted with noradrenaline (1 μM) until three responses of equal amplitude

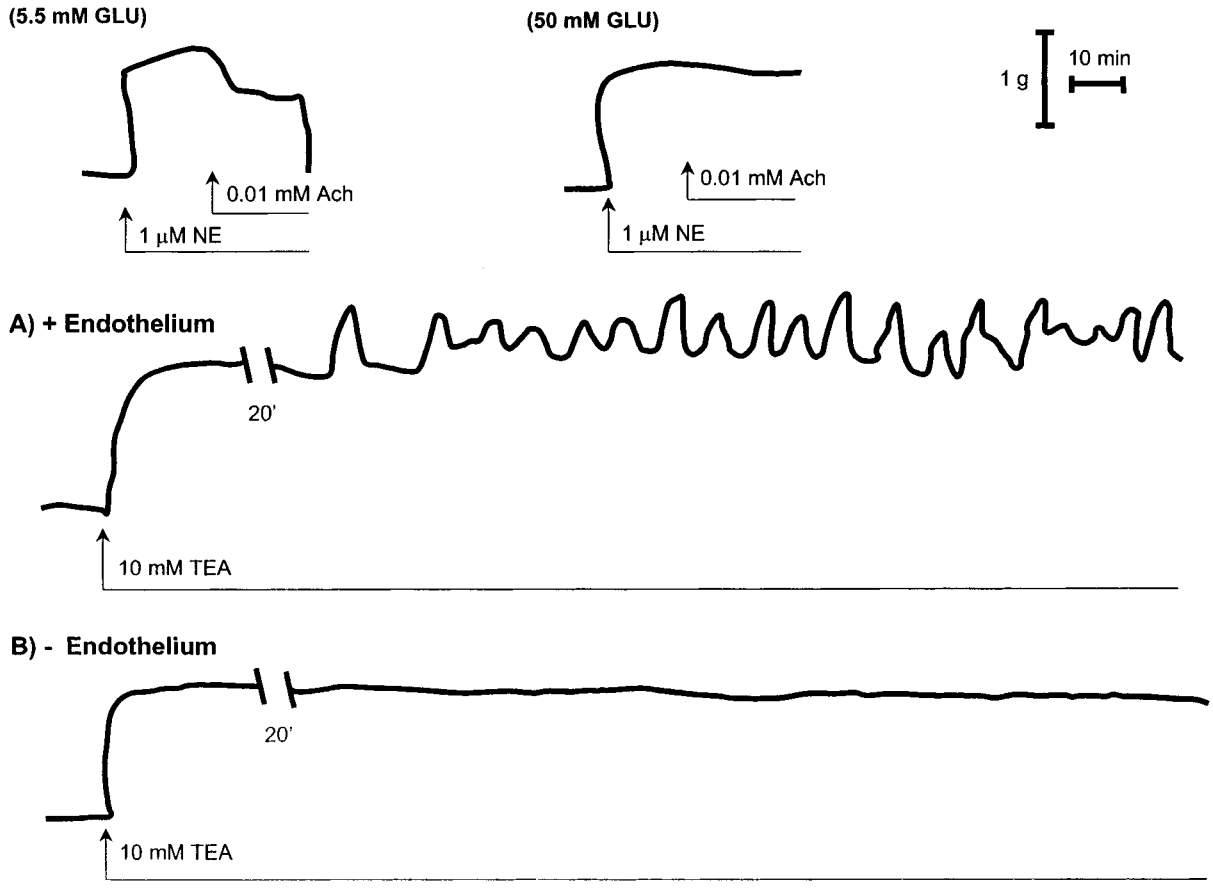

Figure 1 Contractile effects of noradrenaline (NE) and tetraethylammonium (TEA) in guinea-pig aorta rings. The upper two traces were obtained with intact endothelium. The glucose concentration (GLU) in the perfusion medium is indicated. The lower two traces were obtained with (A) and without (B) endothelium; in these two traces, the glucose concentration was 5.5 mM. The tracings are representative of results obtained in nine experiments. Ach: acetylcholine.

were obtained (mean force 5.25±0.35 g, $n=9$). When the contraction stabilized, the response to acetylcholine was tested.

In selected experiments, 3 nM ET-1, 0.1 mM L-arginine or 0.1 mM D-arginine were added to the perfusion medium 30 min before TEA. Where specified, 10 nM verapamil, 1 μM indomethacin, 1 μM apamin, 1 μM glybenclamide, 3 μM metyrapone, 50 nM EMD_{94246} or 6 μM bosentan were added to the perfusion medium during the phasic response of aorta rings to TEA.

Data analysis

Vessel contraction or relaxation induced by the agents under study were reported as per cent change in the force generated by 1 μM noradrenaline. The half-maximal effective concentration (EC_{50}) was estimated graphically as the concentration causing 50% of maximum contraction. Concentration-response curves were constructed cumulatively. Data were expressed as mean±s.e. Statistical evaluation of data was made by means of Student's *t*-test for paired comparisons between responses in rings from the same animal. P values <0.05 were regarded as significant. Where reported, n refers to the number of determinations in different experiments.

Drugs

Noradrenaline bitartrate, tetraethylammonium, acetylcholine chloride, sodium nitroprusside, indomethacin, N^G-nitro-L-arginine methyl ester (L-NAME), L-arginine, D-arginine, apamin, verapamil, metyrapone and glybenclamide were from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). EMD_{94246} was a gift from MERCK-KGaA (Darmstadt, Germany). Bosentan was donated by F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

Results

In the presence of 5.5 mM glucose, endothelium-dependent relaxation was preserved, as shown by the addition of acetylcholine (Figure 1), and persisted after 6 h, as previously demonstrated (Dorigo *et al*., 1997). High glucose concentration (50 mM) abolished the endothelium-dependent relaxation induced by cholinoceptor stimulation (Figure 1). This effect was not due to hyperosmosis, since 50 mM mannose had no effect on acetylcholine-induced relaxation, as we have previously shown (Dorigo *et al*., 1997).

In aorta rings with intact endothelium, TEA initially induced a tonic contraction, followed after 30 min by a vascular spasm characterized by recurrent, long lasting, phasic contractions (Figures 1 and 2). Both the amplitude of the initial response to TEA and the frequency of rhythmic contractions induced by the K^+-channel blocker were concentration-dependent (Figures 3 and 4).

At 0.1 mM, L-NAME, an inhibitor of endothelium-derived relaxing factor (EDRF) synthesis (Muelsch & Brusse, 1990; Moore *et al*., 1990), abolished the phasic response evoked by TEA, unmasking the stable tonic contraction (Figure 2). This effect was always present in preparations responsive to the spasmogenic action of TEA. The inhibitory effect of L-NAME was completely suppressed by preincubation of aorta rings with 0.1 mM L-arginine, the substrate for EDRF synthesis (Muelsch & Brusse, 1990; Calver *et al*., 1991) (Figure 2), but not with the inactive stereoisomer, D-arginine (0.1 mM) (data not shown). When EDRF release was suppressed by removal of endothelium or

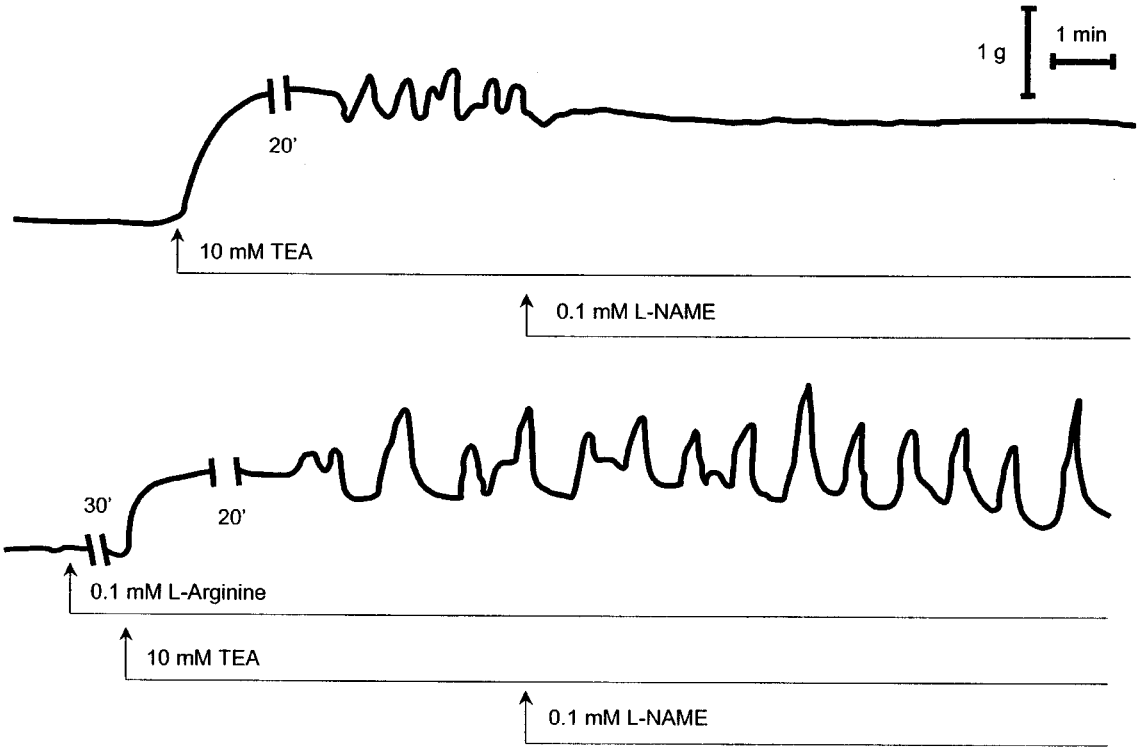

Figure 2 Influence of N^G-nitro-L-arginine methyl ester (L-NAME) on the vascular spasm evoked by tetraethylammonium (TEA) in guinea-pig aorta rings incubated in the absence or in the presence of 0.1 mM L-arginine. Glucose concentration in the medium was 5.5 mM. The effect of L-NAME was evaluated within 1 h after addition of TEA. The tracings are representative of results obtained in ten experiments.

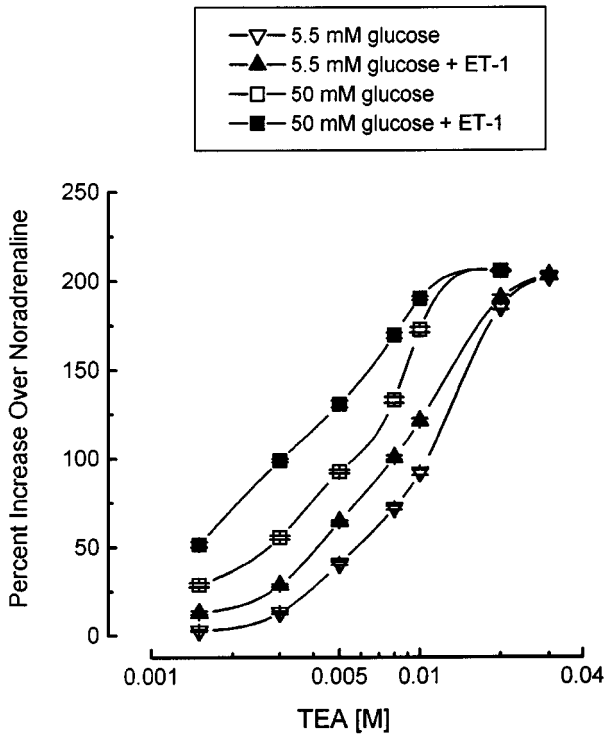

Figure 3 Tonic contraction induced by tetraethylammonium (TEA) in guinea-pig aorta rings: concentration-response relationship. Before addition of TEA, vascular preparations were perfused for 6 h with a medium containing physiologic (5.5 mM) or elevated (50 mM) glucose concentrations. Where indicated, 3 nM endothelin-1 (ET-1) was added 30 min before TEA. The results are expressed as per cent change in the force generated by 1 μM noradrenaline and reported as mean $\pm$ s.e.mean ($n=10$).

by long lasting exposure to elevated glucose, TEA induced only a sustained, concentration-dependent contractile effect (Figures 1, 3 and 4).

ET-1 induced a sustained, concentration-dependent contraction (with a maximum increase of $125.13 \pm 2.41\%$ over noradrenaline-induced contraction, $n=10$). In all the other experiments with ET-1, a concentration of 3 nM was used in order to increase the basal force of contraction by less than 4% and to assure a stable response to TEA.

In aorta rings incubated for 6 h with 5.5 nM glucose (with or without 3 nM ET-1) or 50 mM glucose (with or without 3 nM ET-1), TEA initially evoked a tonic, concentration-dependent contraction. The maximum contractile effect of TEA did not differ under these four experimental conditions (Figure 3). However, 3 nM ET-1, 50 mM glucose and, even more, 50 mM glucose plus 3 nM ET-1, caused a shift to the left in the concentration-response curve of TEA. The EC_{50} of TEA was reduced from 11.39 ± 0.99 mM (in the presence of 5.5 mM glucose; $n=10$) to 7.44 ± 0.44 mM (5.5 mM glucose plus 3 nM ET-1; $n=10$), 5.68 ± 0.27 mM (50 mM glucose; $n=10$) and 3.27 ± 0.17 mM (50 mM glucose plus 3 nM ET-1; $n=10$) (all $P<0.01$ vs 5.5 mM glucose) (Figure 3).

In the presence of 3 nM ET-1, the phasic contractions appeared earlier, within the first 30 min of incubation with TEA, and with increased frequency (Figures 4 and 5). In vessel preparations exposed to elevated glucose, TEA did not induce phasic contractions, unless 3 nM ET-1 was present in the incubation medium (Figure 4). In denuded preparations exposed to elevated glucose, the spasm evoked by TEA in the presence of ET-1 was abolished (Figure 6).

In the presence of intact endothelium, 3 nM ET-1, and 50 mM glucose, the vascular spasm induced by TEA was not modified by 0.1 mM L-NAME, whereas it was amplified by 50 nM EMD_{94246}, an antagonist of ET-1 at ET_A receptors

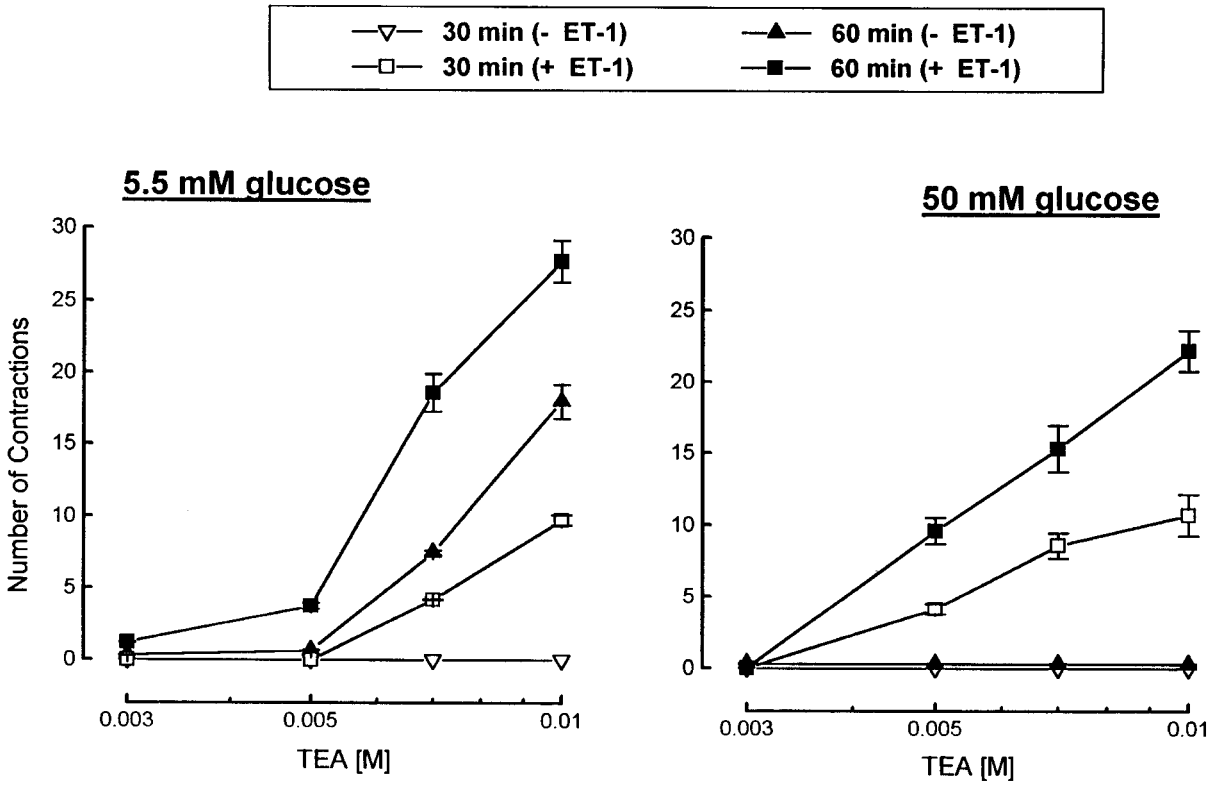

Figure 4 Phasic contractions evoked by tetraethylammonium (TEA) in guinea-pig aorta rings. Before addition of TEA, vascular preparations were perfused for 6 h with a medium containing physiological (5.5 mM) or elevated (50 mM) glucose concentrations. Where indicated, 3 nM endothelin-1 (ET-1) was added 30 min before TEA. The results are reported as mean $\pm$ s.e.mean ($n=6$).

(Osswald *et al*., 1996) (Figure 5). EMD_{94246} did not modify the concentration-effect relationship of TEA-induced tonic contraction (EC_{50} 5.72 ± 0.35 mM; $E_{max} + 208.31 \pm 4.61\%$ over noradrenaline effect; $n = 5$) but prevented the contractile effect of 3 nM ET-1 (Figure 5).

The vascular spasm amplified by EMD_{94246} was abolished by each of the following agents: verapamil, indomethacin, metyrapone, apamin and glybenclamide. These agents suppressed the rhythmic phasic activity and unmasked the tonic contraction. However, only verapamil interfered with the contractile aspect of phasic contractions, whereas all the other inhibitors interfered with the relaxing phase (Figure 5).

The phasic contraction induced by TEA in the presence of 50 mM glucose and 3 nM ET-1 was abolished by the addition of bosentan, an antagonist of ET-1 at ET_A and ET_B receptors (Clozel *et al*., 1994). At 6 μM, bosentan abolished the contractile effect of 3 nM ET-1 (Figure 6), without modifying the concentration-response relationship of TEA (EC_{50}

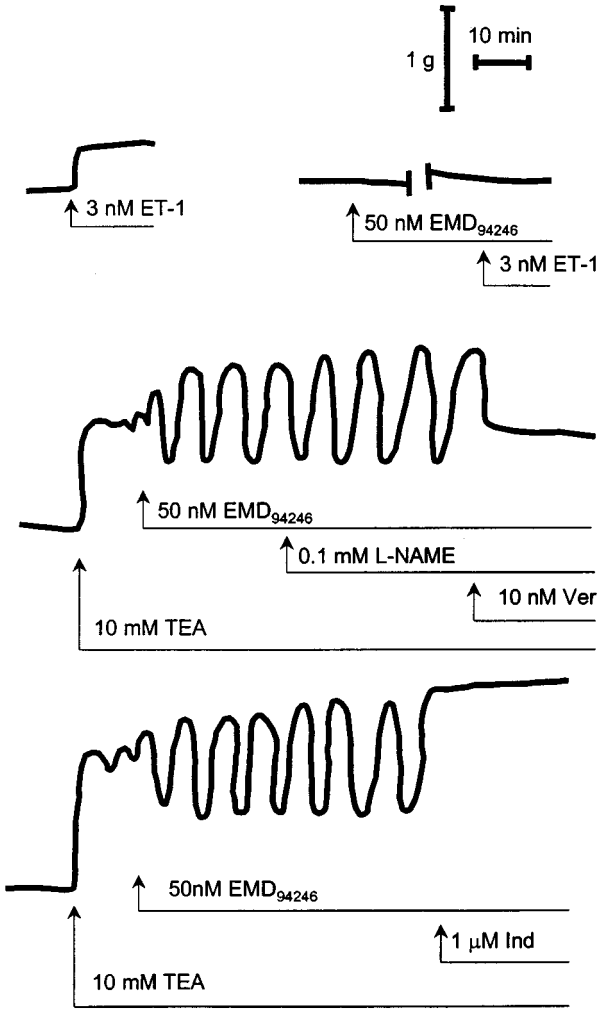

Figure 5 Influence of different agents on the vascular spasm evoked by tetraethylammonium (TEA) in guinea-pig aorta rings. The experiments were carried out in the presence of 50 mM glucose. In the lower two traces, 3 nM endothelin-1 (ET-1) was added to the medium before addition of TEA. The tracings are representative of results obtained in five experiments. In separate experiments, 1 μM apamin, 2 μM metyrapone, or 1 μ glybenclamide, reproduced the effect of indomethacin (as seen in the trace at the bottom). L-NAME: N^G-nitro-L-arginine methyl ester. Ver: verapamil. Ind: indomethacin.

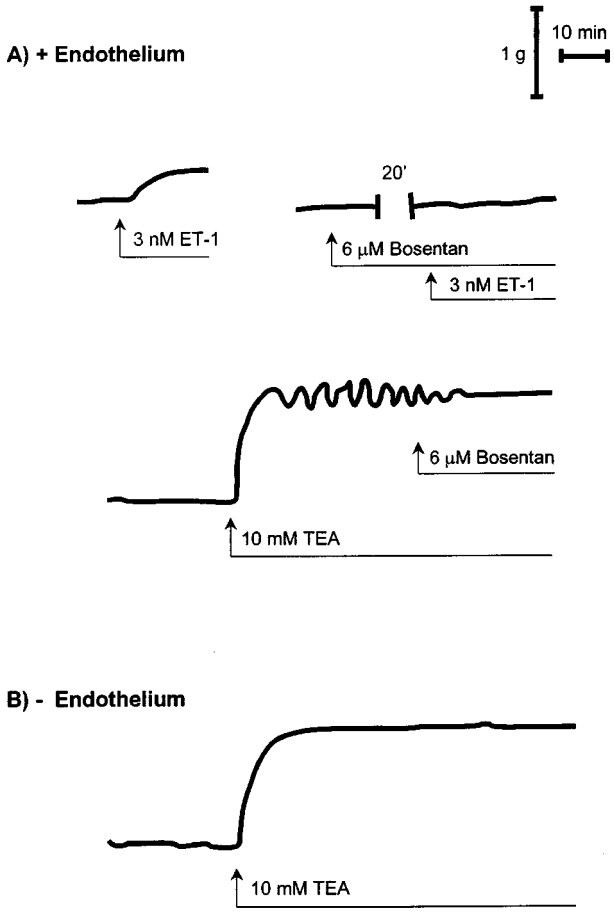

Figure 6 (A) influence of bosentan on the vascular spasm evoked by endothelin-1 (ET-1) and tetraethylammonium (TEA) in guinea-pig aorta rings with intact endothelium. (B) lack of vascular spasm in response to TEA and ET-1 after removal of endothelium. The experiments were carried out in the presence of 50 mM glucose. In the lower two traces, 3 nM ET-1 was added to the medium before addition of TEA. The tracings are representative of results obtained in five experiments.

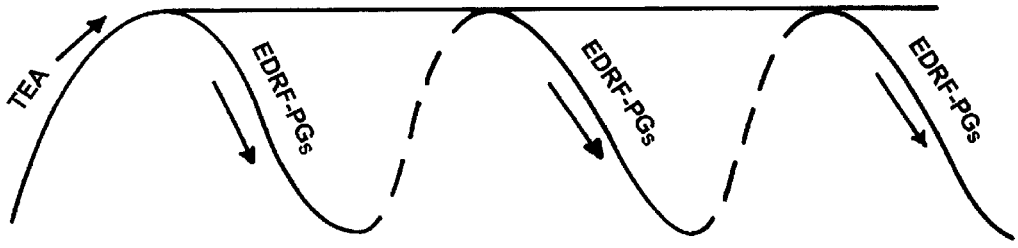

Figure 7 Hypothetical regulatory role of endothelium-derived relaxing factor (EDRF) and prostanoids (PGs) in the genesis and maintenance of vascular spasm evoked by K^+ channel closure under different experimental conditions. TEA: tetraethylammonium.

4.99 ± 0.91 mM; E_{max} + 205.21 ± 6.28% over noradrenaline effect; $n = 5$).

Discussion

Human isolated arteries may exhibit phasic contractile activity, either spontaneously, upon stretch, or after vasoconstricting stimulation. Voltage-operated Ca^{2+} channel antagonists, which act by preventing the opening of the ion channel in response to smooth muscle membrane depolarization, prevent the phasic activity and unmask the stable, tonic contraction in the presence of vasoconstricting stimuli (Weinheimer *et al*., 1983; Sjögren *et al*., 1986; Kimura *et al*., 1989). This indicates that phasic contractions are dependent on Ca^{2+} entry into smooth muscle cells (Ginsburg *et al*., 1984).

Maseri *et al*. (1990) have suggested that segmental arterial spasm is caused by local hyper-responsiveness to a range of vasoconstrictors, although functional synergy may also be involved, as previously shown by the addition of noradrenaline or serotonin to threshold concentrations of ET-1 (Yang *et al*., 1990). However, the mechanisms behind this synergy remain to be elucidated. Moreover, it is still debated whether the prevalent influence of vascular endothelium is towards the genesis and maintenance of vasospasm, through the release of contracting substances, or towards the prevention of vasospasm, by means of relaxing factors.

In the present study, the regulatory role of vascular endothelium in the genesis and maintenance of vascular spasm was investigated. The effects of endothelial integrity and threshold concentrations of ET-1 on the vasoconstricting and spasmogenic effects of TEA were studied in guinea-pig aorta rings. By blocking large conductance K^+_{Ca} channels (Benham *et al*., 1985), TEA induces depolarization in a variety of cells, including vascular smooth muscle (Gillespie & Hutter, 1975; Inoue *et al*., 1989; Tseng & Hoffman, 1989; Bonnet *et al*., 1991), and increases the uptake of Ca^{2+} ions through verapamil-sensitive voltage-dependent channels (Haeusler, 1972; Weiss, 1983).

The vascular spasm induced by TEA in aorta rings was characterized by a recurrent contractile activity superimposed on an initial tonic contraction. The cellular mechanisms involved in TEA-induced phasic spasm are probably different in the presence of intact or damaged endothelium. In fact, in aorta rings with intact endothelium the phasic activity induced by TEA was abolished by L-NAME, an inhibitor of EDRF synthesis, which unmasked the sustained, tonic contraction. The inhibitory effect of L-NAME was prevented by L-arginine, the substrate for EDRF synthesis, but not by its inactive stereoisomer, D-arginine. In contrast, when EDRF release was suppressed by removal of endothelium or by long lasting exposure to high glucose concentrations, TEA induced only a sustained, concentration-dependent contractile effect.

Low concentrations of ET-1 and high concentrations of glucose influenced the tonic and phasic components of vascular contraction. With regard to the tonic response, ET-1, high glucose or both reduced the EC_{50} of TEA, without modifying its maximum contractile effect. Thus, endothelial damage due to elevated glucose sensitized the vascular smooth muscle to the K^+ channel blocker. Low ET-1 concentrations amplified this effect.

With regard to the phasic response, in the presence of intact endothelium, ET-1 reduced the time required for the vasospasm to occur and increased its frequency. In vessels damaged by elevated glucose, TEA induced only a long lasting tonic contraction, unless ET-1 was present, in which case TEA still evoked an initial tonic contraction followed by sustained rhythmic contractions. The phasic spasm induced by TEA in the presence of ET-1 and high glucose concentration was abolished by removal of endothelium or by bosentan, an antagonist of ET-1 at ET_A and ET_B receptors, whereas it was amplified by EMD_{94246}, an ET_A-antagonist. Thus, ET-1 seems to play a permitting role in the phasic spasm evoked by closure of K^+ channels in vessels with intact endothelium or in the presence of high glucose concentration.

In the presence of high glucose and ET-1, the vascular spasm induced by TEA was sensitive to low concentrations of verapamil, which suppressed the phasic response to TEA by abolishing the contractile component of the rhythmic activity, and unmasking the sustained tonic contraction. Since voltage-dependent Ca^{2+}-channels are not present in endothelial cells, verapamil probably interfered by inhibiting Ca^{2+} uptake into smooth muscle cells.

The vascular spasm was also sensitive to apamin, an inhibitor of small conductance K^+_{Ca} channels (Murphy & Braiden, 1995), glybenclamide, an inhibitor of ATP-sensitive K^+ (K^+_{ATP}) channels (Ashcroft, 1988; Cook, 1988), metyrapone, which selectively inhibits cytochrome P-450 (Barber *et al*., 1982; Rossi, 1983), and indomethacin, an inhibitor of cyclo-oxygenase. However, unlike verapamil, these agents abolished the relaxing component of the rhythmic contraction induced by TEA. Therefore, when EDRF release was suppressed by exposure to elevated glucose, the permitting role exerted by ET-1 on TEA-induced vascular spasm was probably sustained by the release of prostanoids, produced by the cytochrome P-450 or the cyclo-oxygenase pathway.

The cytochrome P-450-dependent mono-oxygenase involved in arachidonic acid metabolism is predominantly localized in the endothelium (Abraham *et al*., 1985) and contributes significantly to vasodilatation in different animal species (Cohen & Vanhoutte, 1995; Forstermann *et al*., 1988; MacDonald *et al*., 1988). Cytochrome P-450-dependent metabolites may directly hyperpolarize the smooth muscle cells through activation of different types of K^+-channels (Mombouli & Vanhoutte, 1997). In addition, these metabolites may be further metabolized by the cyclo-oxygenase, to produce hyperpolarizing substances whose action is sensitive to K^+-channel blockers (Mombouli & Vanhoutte, 1997).

Suppression of the relaxing component of rhythmic contraction by glybenclamide and apamin may result from blockade of K^+-channels in smooth muscle cells. However, the effect of glybenclamide and apamin may have resulted from their influence on endothelial cells, as well. In fact, endothelial cells express K^+_{ATP}-channels and small conductance K^+_{Ca}-channels (Nilius *et al*., 1997), which may mediate cell membrane hyperpolarization, leading to the release of endothelium-derived hyperpolarizing factors (Vanhoutte, 1987; Bény & Gribi, 1989; Liu & Flavahan, 1997).

Vessel oscillations induced by TEA seemed to occur mainly when contractions were of intermediate amplitude. This suggests that agents that increase or decrease the tone may prevent oscillations, not by affecting the mechanisms causing oscillations, but by shifting the level of tone to a point where oscillations cannot occur. In fact, although glybenclamide, apamin, metyrapone and indomethacin prevented the phasic spasm by inhibiting the cyclic relaxation, it can not be excluded that these agents increased the basal tone to a level where oscillations cannot occur. However, at variance with this hypothesis, verapamil prevented the phasic contractions without modifying the level of TEA-induced tonic contraction. In addition, EMD_{94246} increased the level of tone reached at

each peak of the rhythmic contraction, without preventing the cyclic oscillations.

These observations suggest that the vascular spasm evoked by TEA in intact guinea-pig aorta originates from the interaction between two mechanisms: the first one, myogenic, is a contraction induced by Ca^{2+} uptake through voltage operated channels in smooth muscle cells, following inhibition of K^+ currents and membrane depolarization (Benham *et al*., 1985); the second one, endothelium-dependent, is a relaxation sustained by the release of EDRF and other vasorelaxing autacoids, probably evoked in a rhythmic manner by contraction itself (Figure 7).

Although rhythmic vessel contractions are ubiquitous throughout the circulation, the mechanisms by which contraction follows relaxation in the course of phasic spasm remain hypothetical. Our study has shown that the endothelium plays a key role in the generation of phasic contractions. Endothelium-derived vasorelaxing autacoids may contribute to the rhythmic contractions by promoting membrane repolarization and relaxation of smooth muscle cells. After relaxation, the smooth muscle can contract in response to depolarizing stimuli, such as blockade of K^+ channels, thus sustaining the cycle of phasic spasm. In fact, as shown in the present study, functional inhibition or removal of the endothelium abolished TEA-induced rhythmic contractility, unmasking a sustained, tonic contraction.

The mechanisms whereby the endothelium synthesizes and releases vasorelaxing autacoids in the course of vascular spasm remain to be established. In guinea-pig aorta rings exposed to TEA, the release of EDRF seemed to follow the vascular contractions. It is unlikely that EDRF is released through a direct influence of TEA on endothelial cells. In fact, EDRF synthesis is a Ca^{2+}-dependent process (Lückhoff *et al*., 1988; Mayer *et al*., 1989), whereas TEA does not increase $[Ca^{2+}]_i$ in endothelial cells. In addition, electrophysiological and unidirectional ^{45}Ca flux measuremnts have shown no voltage-gated calcium channels in these cells (Colden-Stanfield *et al*., 1987; Johns *et al*., 1987; Takeda *et al*., 1987).

The release of EDRF and other vasorelaxing autacoids may be induced by vascular contraction itself (Bansal *et al*., 1993; Wanstall *et al*., 1995). In fact, mechanical forces acting upon endothelial cells, such as fluid shear stress and pulsatile stretch, are important in ensuring the continuous release of vasoactive autacoids (Busse & Fleming, 1997).

A role in the relaxing phase of vascular spasm may also be played by ET-1, through the release of endothelium-derived vasodilating eicosanoids, which interrupt the tonic contraction and give rise to rhythmic phasic activity. In accordance with previous reports, the present study suggests that low concentrations of ET-1 may cause vasodilation through the interaction with endothelial ET_B receptors, which is followed by the release of arachidonic acid metabolites through the cytochrome P-450 and the cyclooxygenase pathways, leading to membrane hyperpolarization of smooth muscle cells (de Nucci *et al*., 1988; Warner *et al*., 1989). ET_{B1} receptors may be involved in this process (Ortega Matgeo & de Artiñano, 1997). A vasodilator activity of ET-1, influenced by glybenclamide, has also been observed *in vivo* (Hasunuma *et al*., 1990; Lippton *et al*., 1991).

Whatever the cellular mechanism involved, it remains to be emphasized that the release of vasodilating agents causes a phasic rhythmic activity during the vascular spasm evoked by TEA in the presence of ET-1 and elevated glucose. This event might be relevant in the arterial spasm of diabetic patients, when hyperglycemia impairs EDRF release (Tesfamariam, 1994) and increases ET-1 plasma concentrations (Yamauchi *et al*., 1990). The present study showed that vascular endothelium plays a prominent regulatory role in the genesis and maintenance of arterial spasm, through the release of vasodilating autacoids.

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