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SIMULTANEOUS OSCILLATIONS IN THE MEMBRANE POTENTIAL OF PIG CORONARY ARTERY ENDOTHELIAL AND SMOOTH MUSCLE CELLS

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SUMMARY

1. The effects of tetrabutylammonium (TBA) on the mechanical tension and on the electrical behaviour of endothelial and smooth muscle cells were studied in intact porcine coronary artery strips.

2. Superfusion of strips with TBA (2–20 mM) induced mechanical oscillations, leading to an increase in tonic isometric tension.

3. TBA-induced mechanical oscillations were correlated with fluctuations of the membrane potential of endothelial cells, which were identified by iontophoretic injection of Lucifer Yellow.

4. The endothelial cell membrane potential fluctuations appeared as action potentials or smaller amplitude slow waves, and were synchronized with electrical membrane potential fluctuations of the underlying coronary smooth muscle cells.

5. Oscillations induced by TBA in smooth muscle cells were not affected by removal of the endothelium, and depended on the presence of calcium in the external medium.

6. To our knowledge, this is the first description of action potential-like fluctuations in the endothelium. It is concluded that the oscillations were generated in the smooth muscle and that they propagate to the endothelium. The question of the mode of propagation of the signal is discussed.

INTRODUCTION

The smooth muscle cells of isolated arteries are usually electrically quiescent. Spontaneous electrical oscillations are not recorded intracellularly, even when strong intra- or extracellular current pulses are applied (Hermsmeyer, 1971; Mekata, 1971, 1974; Mekata & Niu, 1972; Casteels, Kitamura, Kuriyama & Suzuki, 1977; Droogmans, Raeymackers & Casteels, 1977; Harder & Sperelakis, 1978; Ito, Kuriyama & Suzuki, 1978; Kuriyama & Suzuki, 1978; Harder, Belardinelli, Sperelakis, Rubio & Berne, 1979; Holman & Surprenant, 1979). However, when agents like barium or tetraethylammonium, which decrease the conductance to potassium ions, are added to such preparations, membrane potential fluctuations, generally described as action potentials, can be recorded in smooth muscle cells (Mekata, 1971; Droogmans *et al.* 1977; Kumamoto, 1977; Harder & Sperelakis, 1978,

1979; Harder *et al.* 1979; Holman & Surprenant, 1979). These action potentials were correlated with rhythmic changes in arterial tension (Droogmans *et al.* 1977; Kumamoto, 1977; Holman & Surprenant, 1979). Because the above-mentioned studies were performed before the discovery of the role of the endothelium in arterial activity (Furchgott & Zawadzki, 1980), a contribution of the endothelium to the spontaneous activity of smooth muscle was not envisaged and oscillations were generally considered to be myogenic in origin. More recently, the role of the endothelium in oscillatory responses has been questioned. In oscillations induced by histamine in hog carotid arteries (Stein & Driska, 1984) and by noradrenaline in tail arteries of hypertensive rats (Myers, Lamb & Webb, 1985), a role for the endothelium was ruled out. On the other hand, induction of rhythmic contractions by phenylephrine in hamster aortas (Jackson, 1988) and in rabbit mesenteric arteries (Omote, Kajimoto & Mizusawa, 1992) was shown to depend on the presence of functional endothelium. Thus, the contribution of the endothelium to rhythmic contractions is still debated. In addition, rhythmic oscillations of the membrane potential of endothelial cells were recently observed in spontaneously oscillating hamster cheek pouch arterioles (Segal & Bény, 1992). Obviously, the membrane potential behaviour of endothelial cells during electrical oscillations of smooth muscle also needs further investigation.

The purpose of the present study was to investigate the membrane potential behaviour of endothelial and smooth muscle cells of pig coronary artery strips during mechanical oscillations. The pig coronary artery *in vitro* shows no spontaneous mechanical activity and the membrane potentials of smooth muscle and endothelial cells are electrically stable (Ito, Kitamura & Kuriyama, 1979; Bény, Brunet & Huggel, 1986; Bény & Gribi, 1989). We used tetrabutylammonium (TBA), a blocker of calcium-activated potassium channels (Colden-Stanfield, Schilling, Possani & Kunze, 1990; Mendelowitz, Bacal & Kunze, 1992; Rusko, Tanzi, van Breemen & Adams, 1992), to induce mechanical oscillations in these arteries.

METHODS

Preparation of tissues

Left anterior descending branches of coronary arteries were obtained from pigs 10 min after slaughter at an abattoir. The coronary lumen was rinsed by injection of cold (4 °C), oxygenated (95% O_2–5% CO_2) Krebs solution of the following composition (mM): NaCl, 118·7; KCl, 4·7; $CaCl_2$, 2·5; KH_2PO_4, 1·2; $NaHCO_3$, 24·8; $MgSO_4$, 1·2; glucose, 10·1; pH 7·4 at 37 °C. Segments of the coronary artery were dissected out, cleaned of all adherent fat and connective tissue and cut into 2 mm wide rings which were subsequently opened to give strips of about 5 mm in length. In some experiments, the endothelium was removed by gently rubbing the internal surface of the strip with a cotton-tip. The endothelium was considered to be completely absent when the strip did not show any relaxation to 740 nM substance P (SP; Bachem, Feinchemicalen AG, Bubendorf, Switzerland; see Bény *et al.* 1986). In other experiments, the adventitial layer was also removed by separating it from the media using two forceps. All the adventitia plus a small layer of circular smooth muscles were removed leaving only part of the media (Bény, Brunet & Huggel, 1989).

Experimental design

The methods used have been described previously (Bény *et al.* 1986; Bény, 1990; von der Weid & Bény, 1992). Briefly, mechanical tension and transmembrane potential were measured simultaneously. The strip (2 mm wide, 5 mm in length) was incubated in a 100 μl bath and continuously superfused (1250 μl/min) with oxygenated Krebs solution at 37 °C. One extremity of

the strip was firmly pinned (five pins) to the bottom of the chamber, covered with Sylgard (Dow Corning, Ithaca, NY, USA), with its internal (endothelial) side facing up. The other extremity was fixed to an isometric force transducer (Grass FT03C, Quincy, MA, USA). A tension of 10 mN was applied to the strip and when the tension was stabilized (to about 5 mN, after 15 min), preparations

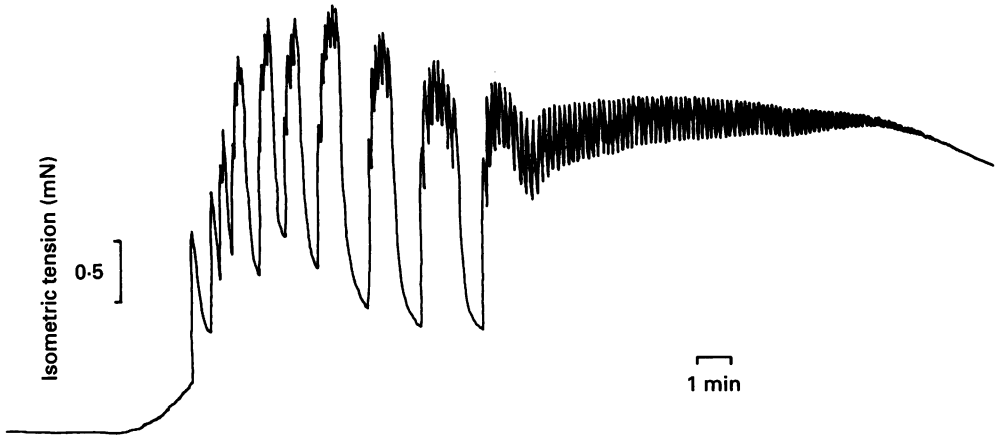

Fig. 1. Typical mechanical oscillatory response of an intact pig coronary artery strip perfused with a TBA-containing Krebs solution. TBA (4 mM) was added 7 min before the beginning of the trace. ACh (10^{-5} M) was present throughout the experiment.

were perfused with tetrabutylammonium (TBA; Sigma, St Louis, MO, USA) diluted in Krebs solution with or without N^G-L-nitro-arginine (100 μM; Aldrich, Steinheim, Germany) throughout the experiment. Acetylcholine (ACh, 10 μM; Sigma) was sometimes added to precontract the strips. Membrane potentials were measured with conventional glass microelectrodes (80–120 MΩ) filled with 3 M KCl. The cells were impaled near the fixed points of the tissue in order to reduce problems associated with muscle movements. Criteria for accepting a record were a sharp drop in potential at cell penetration and withdrawal of the electrode and a stable membrane potential, greater than −40 mV, in the absence of drugs. To impale an endothelial cell, the electrode was gently approached towards the intima until the sudden appearance of a negative potential at the electrode (Northover, 1980; Bény, 1990). To confirm that the recorded cell was indeed from the endothelium, Lucifer Yellow dilithium (5% in water; Sigma), contained in the tip of a conventional microelectrode backfilled with 150 mM LiCl, was microiontophoretically injected by passing a direct current of 3·5 nA (stimulator 215, Hugo Sachs Elektronik, March–Hugstetten, Germany) through the electrode. The tissue was then processed for electron microscopy as described in detail by Bény (1990). Endothelial cell penetration was also accepted when no cell was crossed before the impalement, i.e. no potential deflections were observed either before penetration of the cell, or upon the withdrawal of the electrode (von der Weid & Bény, 1992).

When mechanical tension only was measured to study the involvement of endothelial or adventitial layers on the oscillations, two pairs of strips cut off adjacently from coronary arteries were suspended in four 85 μl baths (Bény *et al.* 1986; Bény, Brunet & Huggel, 1987) and changes in tension were measured as described above.

Experimental data are expressed as means ± standard error of the mean (S.E.M.).

RESULTS

Effect of TBA on the mechanical activity of pig coronary artery strips

Pig coronary artery strips with intact endothelium showed no spontaneous mechanical variation of tension (Ito *et al.* 1979). After 10–20 min in the presence of TBA (2–20 mM), rhythmic contractions leading to an increase in tension appeared in pig coronary artery strips. The concentration of TBA necessary to induce oscillations

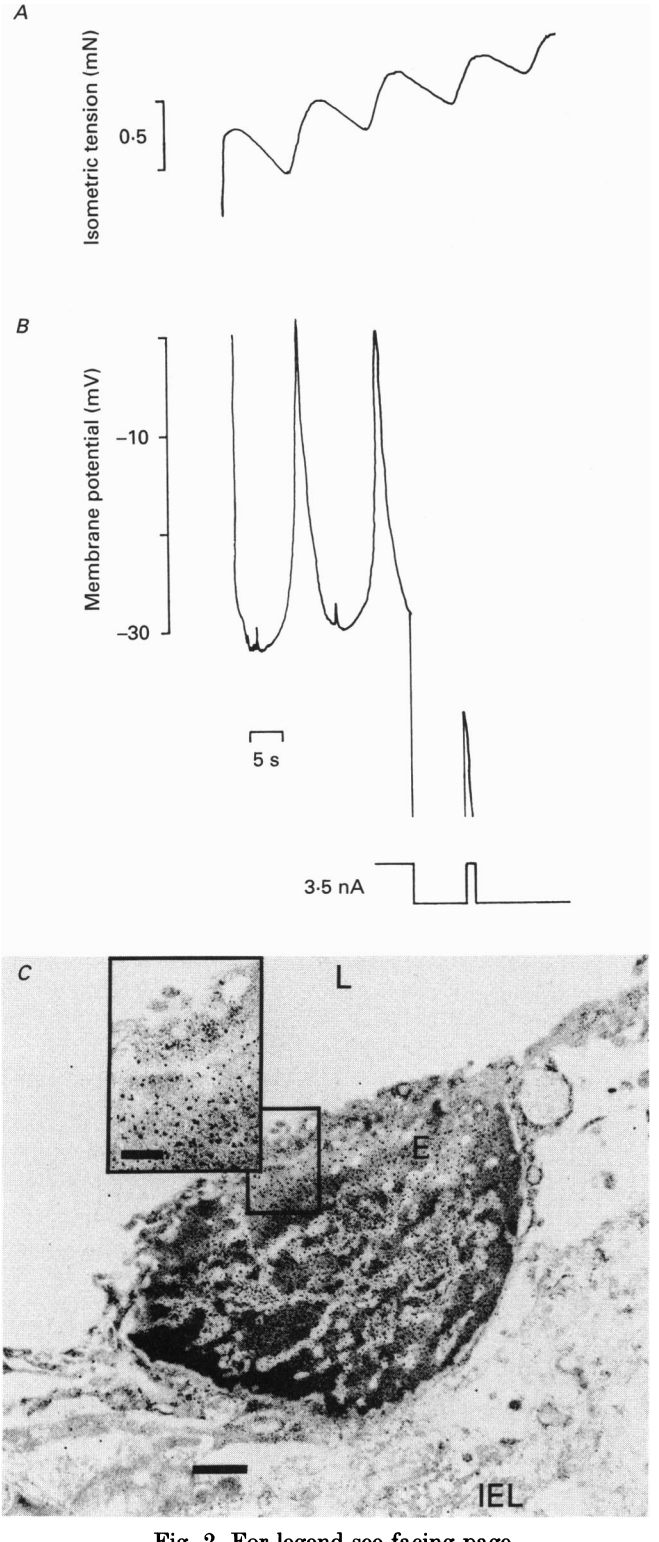

Fig. 2. For legend see facing page.

in thirty-eight strips, dissected from thirty-two pig hearts, varied from one preparation to another. In ten out of the thirty-eight strips, a sustained contraction first appeared, which was replaced by oscillations when the TBA concentration was decreased (to 2 mM), or by washing the preparation with a Krebs solution without TBA. Large variations in the frequency (from 2 to 13 contractions/min; $n = 28$), in the amplitude and in the duration of the rhythmic activity were observed throughout the experiments. After reaching a maximal amplitude, the oscillations usually decreased and stopped while strips remained contracted. Figure 1 shows a typical recording of rhythmic mechanical activity induced by 4 mM TBA. In this experiment, the strip was precontracted with 10 μM ACh, which was added to the incubation medium 3 min before the addition of TBA, but further experiments showed that the presence of ACh was not necessary for the induction of oscillations by TBA (see Figs 2*A*, 3*B* and 4*A*).

Effect of TBA on the electrical activity of endothelial cells

In this series of experiments the membrane potential (E_m) of endothelial cells recorded before or at the very beginning of superfusion of intact strips with a TBA-containing solution was around -47 ± 1 mV ($n = 16$). After 10–20 min in the presence of TBA, a slow depolarization to -32 ± 2 mV ($n = 19$) was first induced and then oscillations of E_m were elicited. These oscillations appeared as fast variations in potential, strongly resembling action potentials, as illustrated in Fig. 2*B*. After slowly (2–3 s) reaching a threshold (-30 ± 2 mV; $n = 14$), E_m then rapidly increased (within 0·6–0·8 s) to a peak (-11 ± 2 mV; $n = 20$). The membrane then repolarized to a value more negative than before the start of firing (-35 ± 2 mV; $n = 17$) and the phenomenon began again with an average frequency of $9{\cdot}6 \pm 0{\cdot}4$ spikes/min ($n = 21$). Sometimes E_m did not reach threshold, and electrical fluctuations of smaller amplitude appeared (Fig. 3). To confirm that the recorded cell was from the endothelium, the dye Lucifer Yellow was injected through the recording electrode as shown in Fig. 2. The labelled cell was identified at the electron microscopic level, by the presence of gold particles coupled to protein A which adhere to the Fc domain of antibodies directed against Lucifer Yellow (Fig. 2*C*).

Simultaneous recordings of endothelial cell membrane potential and strip isometric tension showed that the mechanical and electrical oscillations were precisely synchronized in time (see Figs 2 and 3). Figure 3 shows that endothelial action

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Fig. 2. Simultaneous recordings of mechanical oscillations of a pig coronary artery strip (*A*) and electrical fluctuations from an endothelial cell (*B*) induced by 5 mM TBA. Contractions were correlated with action potential-like signals. During the E_m recording, Lucifer Yellow was microiontophoretically injected by passing a direct hyperpolarizing current of 3·5 nA through the recording microelectrode. This current was briefly interrupted to verify the validity of the recorded membrane potential. The observed hyperpolarizing deflection did not reflect the effective induced potential change, because the potential of this electrode was not compensated. The presence of protein A–gold particles, revealing anti-Lucifer Yellow antibodies, on the transmission electron micrograph (*C*) confirmed the endothelial nature of the E_m recording shown in *B* (magnification × 27 090; bar = 0·5 μm). Inset showed a higher magnification (× 55 440; bar = 0·2 μm) of the surrounded region. L, lumen of the artery; E, endothelial cell; IEL, internal elastic lamina.

potentials were correlated with an increase in mechanical contractions. These contractions started when action potentials reached their peak and then the strip relaxed. As already mentioned above, smaller depolarizations sometimes occurred. They did not reach threshold and were correlated with a small increase in tension.

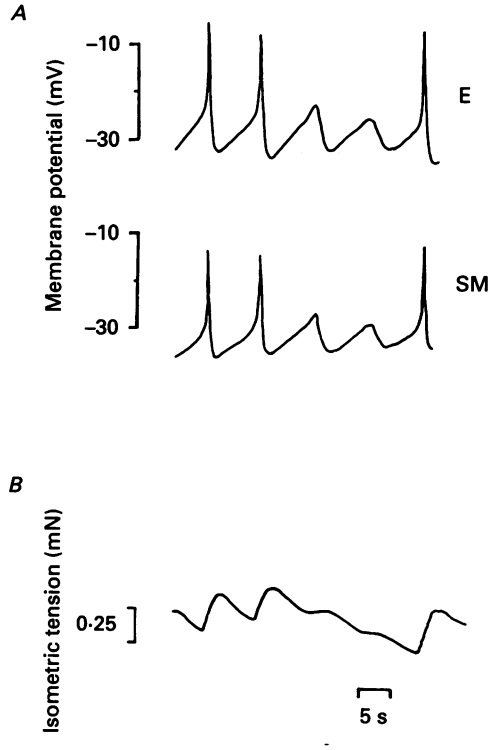

Fig. 3. Simultaneous recordings of membrane potential fluctuations induced by 2 mM TBA in an endothelial cell (E) and in a neighbouring (0·3–0·5 mm) smooth muscle cell (SM), in a strip of pig coronary artery (*A*). The two electrical traces were precisely synchronized. The mechanical activity of the strip was also recorded (*B*). Each action potential-like signal was correlated with a contraction, whereas small electrical fluctuations, which did not reach the threshold, only stopped the relaxation and thus maintained a small tonic contraction.

When the strip was maximally contracted by TBA and no more mechanical oscillations were seen (as shown at the end of the Fig. 1 recording), action potentials disappeared and were replaced by small sinusoidal oscillations (2–3 mV in amplitude). In this condition, perfusion with Krebs solution without TBA relaxed the strip, the cells repolarized and sometimes action potentials appeared again.

Perfusion of SP (740 nM) during E_m oscillations induced by TBA in endothelial cells hyperpolarized the cell, stopped the E_m oscillations and relaxed the strip ($n = 5$).

Effect of TBA on the electrical activity of smooth muscle cells

Before any addition of TBA, smooth muscle cells had recorded E_m values of -46 ± 2 mV ($n = 15$). As in endothelial cells, TBA induced E_m fluctuations in smooth muscle cells. It first depolarized the cell (-32 ± 1 mV; $n = 32$) and then induced

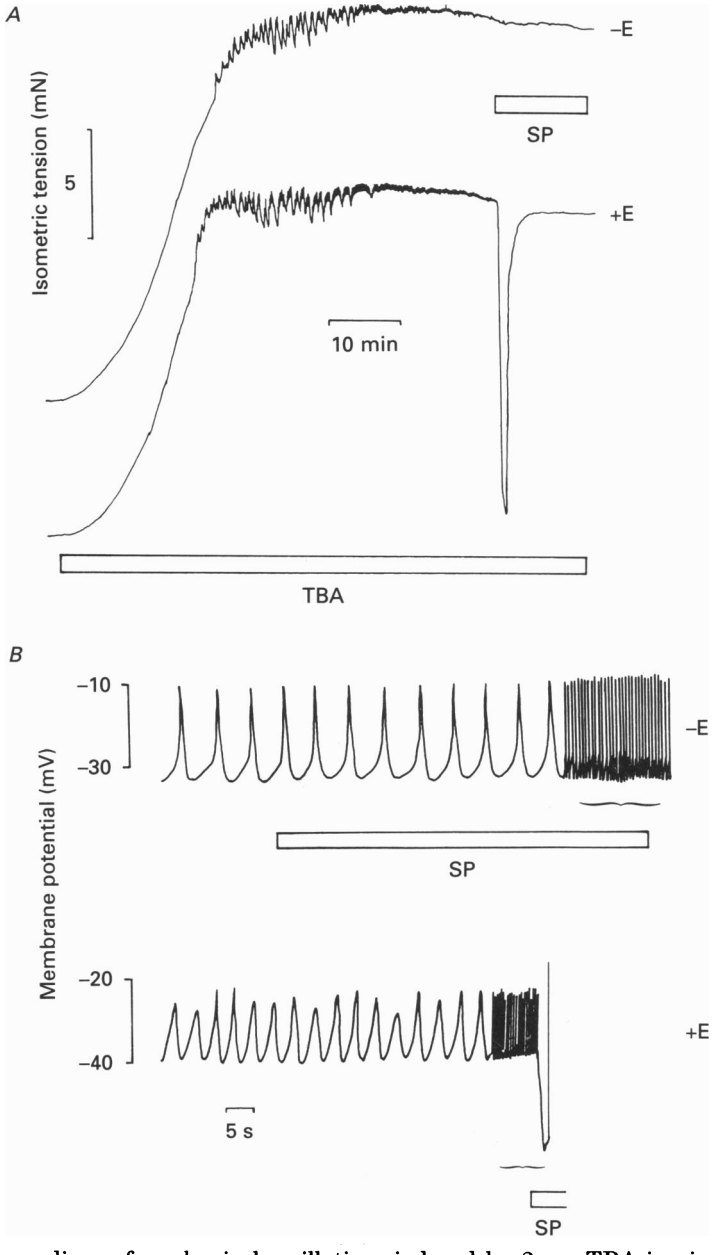

Fig. 4. *A*, recordings of mechanical oscillations induced by 2 mM TBA in pig coronary artery strips without (−E) or with (+E) endothelium in the presence of 100 μM N^G-L-nitro-arginine throughout the recording. *B*, membrane potential fluctuations induced by 2 mM TBA in pig coronary artery smooth muscle cells in the absence (−E) or in the presence (+E) of the endothelial layer. Substance P (740 nM) induced relaxation and hyperpolarization only in the presence of the endothelium.

rhythmic fluctuations which appeared as action potentials, having a threshold of -30 ± 1 mV ($n = 12$) and a maximal depolarization of -14 ± 1 mV ($n = 30$), or as slow waves (maximal depolarization $= -26 \pm 2$ mV; $n = 7$).

Synchronization between endothelial and smooth muscle E_m oscillations

Simultaneous recordings of E_m from an endothelial and a smooth muscle cell were performed by impaling each cell type with a microelectrode. Five double-electrode impalements were successful. One of these is illustrated in Fig. 3. As described above, smooth muscle cell E_m oscillated in the presence of TBA. A precise synchronization existed between endothelial and smooth muscle cell oscillations. Action potentials in the endothelial cell were correlated with action potentials in the smooth muscle cell and slow waves observed in the endothelial cell were recorded at the same time in the smooth muscle cell.

Origin of the oscillations

To investigate the origin of the oscillations, the endothelial cell layer was removed in one of two adjacent strips cut off from four vessels and variations of the isometric tension induced by 2 mM TBA were compared. Oscillations appeared in strips with, as well as without, endothelium. When 100 μM N^G-L-nitro-arginine was added to strips with intact endothelium, to inhibit the synthesis of nitric oxide, the oscillations were not altered ($n = 4$; Fig. 4*A*). Membrane potential oscillations were also induced by 2 mM TBA in smooth muscle cells of de-endothelized strips ($n = 4$; Fig. 4*B*). The absence of the endothelium was confirmed by the loss of endothelium-dependent relaxing and hyperpolarizing responses to SP (Bény *et al.* 1986) in the smooth muscle cell. As a control, the usual relaxing and hyperpolarizing responses to SP were recorded in the presence of intact endothelium (+E, Fig. 4*A* and *B*).

To test whether the oscillations are neurogenic, the adventitial layer was removed in some strips, and variation of the isometric tension was compared with adjacent strips, where the adventitia was left intact. In these conditions, TBA induced contractions in all of the nine adventitia-devoided strips tested, but the contraction was in each case smaller than that induced in the corresponding adjacent adventitia-containing strip. Mechanical oscillations were observed in eight strips with intact adventitia and in six of the eight adjacent strips devoid of adventitia (not shown).

External calcium dependence of the TBA effect

TBA-induced contraction and mechanical oscillations disappeared when strips with intact endothelium were superfused with a calcium-free Krebs solution containing 2 mM EGTA ($n = 6$).

DISCUSSION

In the present study, mechanical oscillations were induced in pig coronary artery strips when TBA was added to the perfusing Krebs solution. These mechanical events were correlated with membrane potential fluctuations of endothelial and smooth muscle cells. Electrical fluctuations appeared either as action potentials or slow waves of smaller amplitude. To our knowledge, this is the first description of an action potential-like signal in endothelial cells. However, small rhythmic variations of cultured endothelial cells, E_m have been mentioned in recent studies (Mehrke & Daut, 1990; Laskey, Adams, Cannell & van Breemen, 1992).

TBA has been described as a blocker able to suppress the Ca^{2+}-activated K^+ current induced by bradykinin in bovine aortic endothelial cell monolayers (Colden-Stanfield *et al.* 1990; Mendelowitz *et al.* 1992; Rusko *et al.* 1992). However, to our surprise, in our preparations hyperpolarizations induced by SP in endothelial cells were not affected by the application of TBA. This seems to indicate that SP and bradykinin activate different channels, or that bovine aortic endothelial cells in culture behave differently from pig coronary endothelial cells *in situ*. But in the three above-mentioned studies, oscillations in endothelial cell membrane potential in response to TBA were not described. In fact, our results obtained with intact strips do not demonstrate that TBA directly induced oscillations in pig coronary endothelial cells. Since the endothelial and smooth muscle cell E_m fluctuations were always perfectly synchronized, it is difficult to believe that they could be caused by independent yet simultaneous actions of TBA on these two cell types. Moreover, TBA-induced electrical oscillations still occurred in smooth muscle cells when the endothelium was rubbed off or when N^G-L-nitro-arginine was added to the Krebs solution, ruling out an endothelium-dependent mechanism. In addition, the contractions and mechanical oscillations we observed in strips devoid of adventitia seem to exclude a neurogenic origin for the oscillations. Taken together, these data suggest a muscular origin of the oscillating response to TBA, which is instantaneously transmitted to the endothelium. While most of the electrical and mechanical oscillations described so far in arteries appear also to have a muscular origin, endothelium-dependent agonist-induced rhythmic contractions were observed in hamster aortae (Jackson, 1988). This result does not contradict the endothelium-independent oscillations we describe here, as it was shown that the hamster aortic endothelium did not itself generate the oscillating signal, but that the continuous release of nitric oxide induced an accumulation of cGMP in smooth muscle cells (Jackson, Mülsch & Busse, 1991), which presumably caused a perturbation of the calcium homeostasis in smooth muscle cells, leading to the mechanical oscillations. Thus the oscillations in hamster aorta were endothelium dependent but myogenic.

As indicated by the present results, the contractile rhythmicity seems to be generated by action potential-like fluctuations of smooth muscle E_m. Although this aspect of the question was not investigated in this study, the suppression of mechanical oscillations we observed, when calcium was removed from the superfusing solution, suggests that calcium ions are involved in these oscillations (Janssen & Daniel, 1991); however, further investigations are needed to determine precisely the role of these ions in this mechanism.

Although transmission of information from the endothelium to the underlying smooth muscle is well documented (for review see Rubanyi, 1991), transmission of signals from the smooth muscles to the endothelial cell layer is quite a new concept. Two hypothetical transmission modes may be proposed: paracrine transmission of a diffusible muscular factor or electrotonic spreading by electrical coupling with or without regeneration of the signal.

The precise time correlation between smooth muscle and endothelial cell electrical fluctuations shown in the present study is incompatible with the action of a diffusible muscular factor, which must be released and act instantaneously on the endothelium. Induction of fast endothelial cell E_m fluctuations by such a muscular factor would

suggest the involvement of voltage-activated ionic channels. Voltage-activated ionic channels would also be involved if the electrical signal transmitted from the smooth muscle is regenerated in endothelial cells. The existence of voltage-dependent calcium channels was recently demonstrated by Bossu, Elhamdani & Feltz (1992) in confluent bovine capillary endothelial cells. But in other endothelial cells, voltage-dependent calcium entry was not observed (Colden-Stanfield, Schilling, Ritchie, Eskin, Navarro & Kunze, 1987; Johns, Lategan, Lodge, Ryan, van Breemen & Adams, 1987; Takeda, Schini & Stoeckel, 1987).

Alternatively, electrical signals could be transmitted from the smooth muscle to the endothelium by pure electrotonic spreading. Electrotonic spreading would occur via gap junctions between endothelial and smooth muscle cells, which are probably localized, in arteries, at the level of the myoendothelial bridges (Thoma, 1921; Spagnoli, Villaschi, Neri & Palmieri, 1982; Davies, 1986); however, the existence of gap junctions at this level is controversial. For example, close apposition of endothelial and smooth muscle cells was observed in the dog coronary artery, but without fusion of membranes of both cells (Berne & Rubio, 1979). Furthermore, we demonstrated a lack of Lucifer Yellow dye coupling between endothelial and smooth muscle cells in pig coronary arteries (Bény, 1990). This is in apparent opposition to the hypothesis of an electrotonic transmission between smooth muscles and the endothelium. To solve this apparent contradiction, we hypothetized that gap junctions were too scarce to allow the transfer of dye between the smooth muscle and the endothelial cells, and that electrical signals can only spread from the smooth muscle syncytium to the unistratified endothelial cells, but not in the opposite direction, i.e. from the endothelium to the underlying smooth muscle. This concept is biophysically tenable since it would be easy for a membrane potential change of the enormous membrane area of the smooth muscle syncytium (i.e. the low input resistance of the coupled smooth muscle cells compared to that of the endothelium) to supply enough local circuit current across an electrical junction to elicit, without regeneration by specific voltage-gated channels, a significant membrane potential change in the comparatively small endothelial layer, because of its small membrane area (i.e. the high input resistance). This hypothesis thus implies that the electrotonic transmission of information from the smooth muscle to the endothelial cells would be unidirectional. A classical example of such unidirectional electrical conduction was described in the nerve cord of the crayfish, where conduction occurs from a large axon to a small crossing nervous fibre through gap junctions, but not in the other direction (Furshpan & Potter, 1959). This hypothesis of unidirectional electrotonic transmission implies a different mechanism for the transmission of information from the endothelium to the media, which is compatible with the existence of diffusible endothelial factors like endothelium-derived relaxing factor (EDRF), prostacyclin, endothelin and putative endothelium-derived hyperpolarizing factor (EDHF).

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