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Factors inducing endothelium-dependent relaxation in the guinea-pig basilar artery as estimated from the actions of haemoglobin

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1 Factors inducing dilatation of guinea-pig basilar artery were investigated in intact and endothelium-denuded tissues by measurement of isometric tension and by electrophysiological methods.

2 The amplitudes of contractions induced by 9,11,epithio-11,12-methanothromboxane A_2 (STA_2) and by high K^+ were enhanced by haemoglobin (oxyhaemoglobin, Hb) in a concentration-dependent fashion (above 1 μM). For the high K^+-induced contraction, the initial tonic component was enhanced to a greater extent than the secondary phasic component. Mechanical responses evoked by STA_2 and by high K^+ were greater in endothelium-denuded tissues, but Hb (below 10 μM) had no effect on them.

3 Hb (10 μM) had no effect on the contractile proteins as estimated from the actions of Hb on Ca^{2+}-induced contractions in skinned muscle tissues. Further, Hb had no effect on the release of Ca^{2+} from intracellular stores but it accelerated the Ca^{2+} accumulation into the sarcoplasmic reticulum as judged from the caffeine- or STA_2-induced contraction generated in intact tissues.

4 Acetylcholine (ACh) relaxed tissues that were precontracted by STA_2 but Hb prevented this relaxation, in a concentration-dependent fashion. The ACh-induced relaxation was sustained for over 10 min in the absence of Hb, but following application of Hb, ACh caused only a transient relaxation.

5 STA_2 (up to 100 nM) did not modify the resting membrane potential of smooth muscle cells of the basilar artery. ACh (10 μM) caused transient hyperpolarization which was only slightly inhibited by Hb (10 μM) whether or not STA_2 was present. The hyperpolarization induced by ACh required the presence of endothelial cells.

6 A23187 (0.01–1 μM) relaxed tissues which were precontracted by STA_2, in a concentration-dependent fashion but had no effect on the membrane potential.

7 These results suggest that in guinea-pig basilar artery, ACh induces relaxation of tissues that were precontracted by STA_2 by causing release of both endothelium-derived relaxing (EDRF) and endothelial dependent hyperpolarizing factor (EDHF) (sustained and initial transient relaxation, respectively), but via different mechanisms. Hb inhibits the former and to a lesser extent, the latter. Since A23187 produced relaxation of pre-contracted tissue but caused no detectable change in the membrane potential, this agent may release EDRF but not EDHF.

Introduction

Furchgott & Zawadzki (1980) demonstrated that the vasodilator action of acetylcholine (ACh) on the rabbit aorta was mediated indirectly by release from endothelial cells of a relaxant substance which they later termed endothelium-derived relaxing factor (EDRF) (Cherry *et al.*, 1982). The action of EDRF is associated with a rise in cyclic GMP synthesized in the cytosol of vascular smooth muscle cells (Holzmann, 1982; Rapoport & Murad, 1983; Diamond & Chu, 1983; Furchgott & Jothianandan, 1983; Ignarro *et al.*, 1984) and this stimulation appears to be inhibited both by haemoglobin (Hb) and myoglobin but not by methaemoglobin (Miki *et al.*, 1977; Murad *et al.*, 1978; Mittal *et al.*, 1978). Furthermore, both the endothelium-dependent relaxation and the rise in cyclic GMP induced by ACh in

bovine coronary and intrapulmonary arteries are blocked by methylene blue (Holzmann, 1982; Ignarro et al., 1984; Martin et al., 1985). Recently Komori & Suzuki (1987a,b) reported that in the rabbit saphenous artery ACh and oxotremorin release EDRF through activation of muscarinic M_2-receptors, and the former but not the latter induces an endothelial-dependent hyperpolarization by releasing a hyperpolarizing substance which we propose to term EDHF (endothelial-dependent hyperpolarizing factor) through activation of muscarinic M_1-receptors. Hb inhibited the relaxation to a greater extent than the hyperpolarization (Chen & Suzuki, 1988). Such endothelium-dependent hyperpolarizations induced by agonists have been reported (Bolton et al., 1984; Bolton & Clapp, 1986; Southerton et al., 1987; Feletou & Vanhoutte, 1988).

Although the action of EDRF on various vascular smooth muscle cells was inhibited by Hb through inhibition of guanylate cyclase, Hb itself or haemolysate produces contraction of basilar arteries in many species (Tanishima, 1980; Toda et al., 1980; Wellum et al., 1982; Fujiwara & Kuriyama, 1984; Fujiwara et al., 1986; Connor & Feniuk, 1987). Thus, the vasoconstriction that occurs after subarachnoid haemorrhage following rupture of cerebral aneurysms may be related to the actions of Hb (Saito et al., 1977; Fisher et al., 1980; Wellum et al., 1982; Kassel et al., 1985; Boullin, 1985).

The present experiments were intended to clarify some of the features of endothelium-dependent relaxation in the guinea-pig basilar artery by use of microelectrode and tension recording techniques in intact and endothelium denuded tissues.

Methods

Guinea-pigs of either sex (250–350 g) were killed by decapitation, taking care to avoid subarachnoid haemorrhage. After craniotomy the basilar artery with brain stem was removed and placed in a small chamber filled with Krebs solution. After dissecting the basilar artery, the arachnoid membrane and connective tissue were carefully removed. Under binocular microscope, circularly cut strips (0.03–0.05 mm in thickness, 0.3–0.5 mm in length and 0.1 mm in width) were prepared for recording tension, and whole muscle tissues (0.2–0.3 mm in diameter and 5 mm in length of vascular tissues without dissection) were prepared for recording membrane activity. To remove the endothelium, the intimal surface was gently rubbed with small knives made from pieces of razor blade (Kanmura et al., 1987). Satisfactory ablation of the endothelium was histologically verified under a light microscope.

Solution

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134.4, and glucose 11.5. The solution was bubbled with 97% O_2 and 3% CO_2, and the pH was adjusted to 7.4. High K^+ solution or Ca^{2+}-free solution was prepared by isoosmotic replacement of NaCl with KCl or of $CaCl_2$ with $MgCl_2$ (with 2 mM EGTA), respectively.

Recording of mechanical activity

The circularly cut strip was set up in a small 1.0 ml chamber, through which the test solution could be changed rapidly by injecting fresh solution from one end and by sucking simultaneously from the other end with the water pump. Both ends of the preparation were fixed between pieces of Scotch double-sided tape via thin silk, and isometric tension was recorded with a strain gauge transducer (U-gauge, Shinko Co.). The temperature of the perfusate was kept at 25°C. All experiments were started after at least 90 min equilibration, under a resting tension of approximately 0.5 mg.

To record the Ca^{2+}-induced contraction in skinned muscle tissues, tissues were exposed to saponin (40 $\mu g\,ml^{-1}$) for 20 min in relaxing solution (mM; K-methanesulphonate 90, piperazine-N-N'-bis-(2-ethanesulphonic acid) (PIPES) 20, Mg-methansulphonate 5.1, ATP 5.2 and EGTA 4: Itoh et al. 1982) at 25°C. The tension-pCa relationship was obtained by cumulative application of increasing Ca^{2+} concentrations buffered by EGTA (Itoh et al., 1982) in a stepwise manner. Hb was applied during the Ca^{2+}-induced contraction after tension had reached a steady level.

Recording of membrane activity

The preparation was mounted in an organ bath with a capacity of 1 ml and then superfused with warmed Krebs solution (35°C) at a flow rate of about 2 $ml\,min^{-1}$. The experiments were carried out after 120–150 min of superfusion. Electrical responses of the membrane were recorded via a glass capillary electrode (Higenberg Glass) filled with KCl (3 M). The resistance of the electrode tip ranged between 40–60 MΩ. The microelectrode was inserted from the outer surface of the artery.

Drugs

Oxyhaemoglobin was prepared by reducing commercial bovine Hb containing 75% methaemoglobin (Sigma) as described by Martin et al. (1985). Briefly

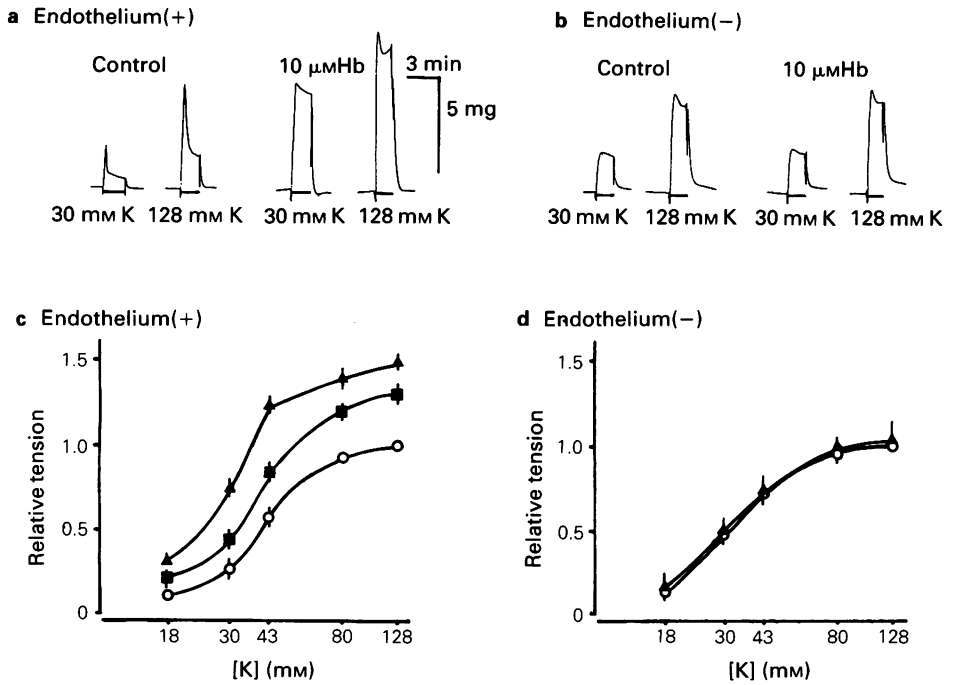

Figure 1 Effects of oxyhaemoglobin (Hb) on contractions evoked by various concentrations of K^+ in the presence (a and c) or absence of endothelial cells (b and d). Effects of Hb (10 μM) on the individual contractions evoked by K^+ (30 mM and 128 mM) in intact tissues (a), or in the endothelium-denuded tissues (b). Grouped data showing effects of Hb (1 and 10 μM) on K^+-induced phasic contraction in intact ($n = 5$) and endothelium-denuded ($n = 4$–6) tissues are shown in (c) and (d). The amplitude of the contraction evoked by K^+ (128 mM) in intact tissues was normalised as a relative value of 1.0: (○) control; (▲) 10 μM Hb; (■) 1 μM Hb.

after adding 10 times sodium dithionite to Hb (1 mM), dialysis by 200 times distilled water was carried out at 4°C. The identification and final concentration of oxyhaemoglobin were determined spectrophotometrically. Hereafter the term Hb is used to mean oxyhaemoglobin. The chemicals used were acetylcholine Cl (Daiichi), saponin (ICN Pharmac. Co.) 9,11-epithio-11,12,-methano-thromboxane A_2 (STA_2; Ono Pharmac. Co.), nifedipine (Bayer Pharmac. Co.), caffeine (Wako Pure Chem.), A23187 (calimycin, free acid; Sigma) and ethyleneglycol-bis (β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA, Dozin Pharmac.).

Statistics

The measured values are expressed as the mean ± s.d. and accompanied by the number of observations. Statistical significance was assessed by Student's t test. P values less than 0.05 were considered significant.

Results

Excitatory effects of haemoglobin on the K^+- and STA_2-induced contractions in intact and in endothelium-denuded tissues

Hb itself did not produce any detectable contraction on muscle strips prepared from the guinea-pig basilar artery. All of the studies described here relate to the effects of Hb on the pharmacological action of other agents. In endothelium-intact tissues, application of K^+ (30 mM) produced a rapid (phasic) contraction followed by a low amplitude (tonic) contraction. The maximum amplitude of contraction that could be induced by K^+ was evoked by a concentration of 128 mM. However, when Hb (10 μM) was applied 10 min before and during application of high K^+ solutions, the amplitudes of both the phasic and tonic contractions were markedly enhanced (Figure 1a). When the amplitude of the phasic contraction evoked by 128 mM K^+ in the absence of Hb was normalized as a relative tension of 1.0, Hb

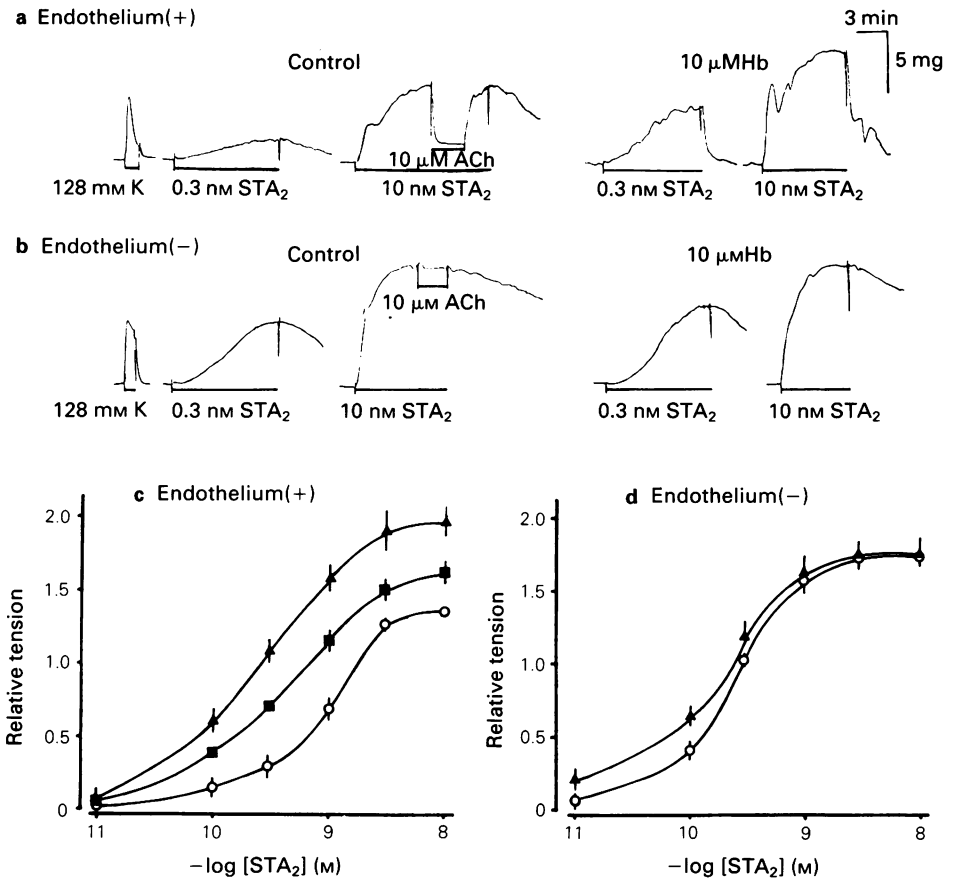

Figure 2 Effects of oxyhaemoglobin (Hb) on the STA_2-induced contraction in intact and endothelium denuded tissues. (a) Examples of contractions evoked by STA_2 (0.3 nM and 10 nM) in the presence or absence of Hb (10 μM) in intact tissues. The contraction evoked by K^+ (128 mM) was taken as the control. (b) The same experimental protocol as in (a), following removal of the endothelium. In (a) and (b), acetylcholine (ACh, 10 μM) was applied during the STA_2-induced contraction to establish whether the endothelium had been removed from the tissue. (c and d) Grouped data showing the effects of Hb on the STA_2-induced contraction before ($n = 3$–8) and after ($n = 4$–6) removal of endothelial cells. The contraction amplitude evoked by K^+ (128 mM) in intact tissues was normalized as 1.0: (○) control; (▲) in the presence of Hb (10 μM) and (■) Hb (1 μM), respectively. Hb was applied 10 min before application of STA_2.

(above 1 μM) consistently enhanced the phasic contraction amplitude at any given concentration of K^+, in a concentration-dependent fashion (Figure 1c).

Following removal of endothelium, the tonic contraction evoked by high K^+ solutions (30 mM and 128 mM) was markedly enhanced (Figure 1b) and reached virtually the same amplitude as the phasic contraction. Any enhancement of the phasic contraction amplitude relative to that observed in intact muscle tissues was minimal. Application of Hb (10 μM) had no effect on the contraction amplitude (Figure 1b). In fact, Hb had no effect on the contractile amplitude in endothelium-denuded tissue at any concentration of K^+ (Figure 1d).

Application of STA_2 (0.3 nM or 10 nM) induced contraction, and application of STA_2 (10 nM) induced a contraction of much higher amplitude than that induced by K^+ (128 mM) and the relaxation of the tissue after removal of STA_2 was very much slower than after removal of K^+ (Figure 2a). Application of Hb (10 μM) enhanced the STA_2-induced contraction (Figure 2a). In endothelium-denuded tissues, the amplitude of the contraction provoked by STA_2 was enhanced compared to that observed in intact tissues, but application of Hb (10 μM) then produced

no further enhancement of the contraction amplitude (Figure 2b). To ascertain whether the endothelium was removed, the effect of ACh (10 μM) was examined (compare Figure 2a and b); the results showed that ACh caused relaxation only in the intact tissue. In intact muscle tissues, Hb (1 and 10 μM) enhanced the contraction evoked by STA_2 in a concentration-dependent fashion (Figure 2c). However, in the endothelium-denuded tissue (Figure 2d), the contraction amplitude was slightly enhanced by Hb only at low concentrations of STA_2 (below 1 nM).

Effects of Hb on the STA_2-induced contraction in Ca^{2+}-free solution or in the presence of Ca antagonist

The effects of Hb on STA_2-induced contractions in intact muscle tissues in Ca^{2+}-free solution or in the presence of nifedipine were also studied (Figure 3). After removal of Ca^{2+} from the Krebs solution containing EGTA (2 mM) for 7 min, STA_2 (10 nM) produced a small amplitude contraction and re-addition of Ca^{2+} (2.6 mM) restored the contraction amplitude to that observed in the presence of Ca^{2+}. When Hb (10 μM) was applied 5 min before application of STA_2 in Ca^{2+}-free solution, the amplitude of the contraction evoked by STA_2 (10 nM) was not affected, but that evoked by STA_2 following re-addition of Ca^{2+} (2.6 mM) was enhanced. When Ca^{2+} was removed again, the tissue relaxed rapidly (Figure 3a). The effects of Hb on the STA_2-induced contraction during treatment with nifedipine are shown in Figure 3b. Since nifedipine (0.3 μM) blocked the K^+-induced contraction in guinea-pig basilar arteries which were endothelium-denuded (Nishiye *et al.* 1988), this same concentration of nifedipine was applied for 10 min before application of STA_2 (10 nM). Nifedipine reduced the contraction amplitude to half of the control, but pre-application of Hb (10 μM) restored the contraction amplitude evoked by STA_2 (10 nM) so that there was only a slight reduction in the amplitude compared with that observed in the absence of nifedipine. Furthermore, ACh (10 μM) inhibited the contraction evoked by STA_2 (10 nM) to 0.6 times the control in the presence of nifedipine (0.3 μM; $n = 3$). The extent of this inhibition was roughly the same as observed in the absence of nifedipine.

Effects of Hb on the caffeine-induced contraction

To investigate the actions of Hb on intracellular Ca^{2+} stores, the effects of Hb on the caffeine-induced contraction were observed in intact tissues in Ca^{2+}-free solution. As shown in Figure 4a, the first application of caffeine (2 mM) produced a transient contraction. A second application of caffeine (2 mM) given immediately after the first contraction had little effect (not shown), but application of caffeine (20 mM) after an interval of 5 min, produced a contraction that was small in comparison to that evoked

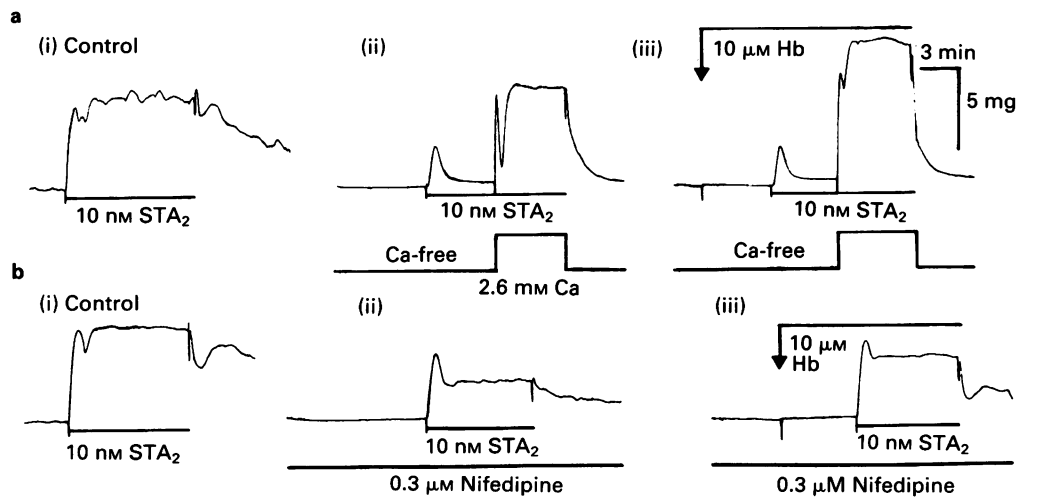

Figure 3 Effects of oxyhaemoglobin (Hb) on the STA_2-induced contraction in intact tissues in Ca^{2+}-free solution containing EGTA (2 mM) or in the presence of nifedipine. (a) (i) Contraction induced by STA_2 (10 nM) was recorded as the control. (ii) Seven min after transition to Ca^{2+}-free solution STA_2 (10 nM) was applied, then Ca^{2+} (2.6 mM) was applied for 5 min. (iii) The same protocol as in (ii) but 5 min before application of STA_2, Hb (10 μM) was added. (b) The response to STA_2 (10 nM; control, (i)), in the presence of nifedipine (0.3 μM), applied 10 min before application of STA_2, (ii), and with the addition of Hb (iii).

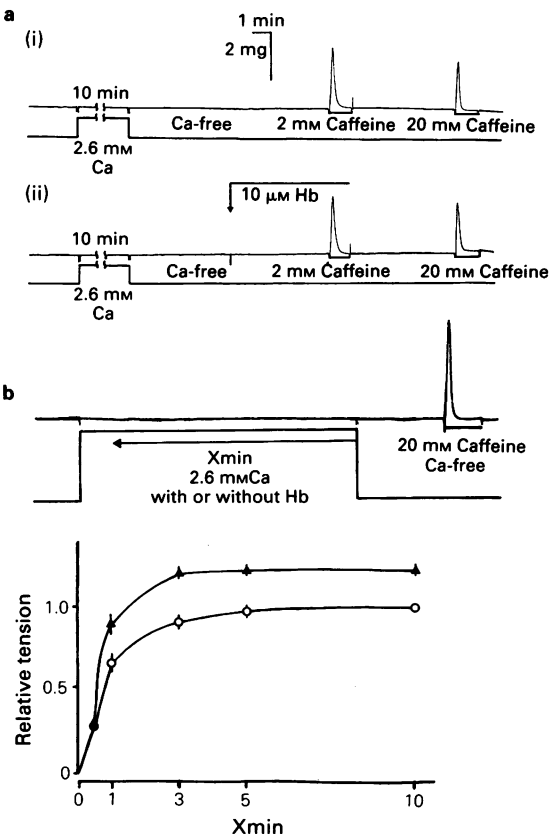

Figure 4 Effects of oxyhaemoglobin (Hb) on the Ca^{2+} release from and Ca^{2+} accumulation into intracellular storage sites as estimated from the amplitude of caffeine-induced contraction in intact tissues. (a) (i) After a period in Ca^{2+}-free solution so as to deplete cellular Ca^{2+} stores, Ca^{2+} (2.6 mM) was applied for 10 min and then the Ca^{2+}-free solution was restored. Ten min later caffeine (2 mM then 20 mM) was applied with an interval of 5 min. (ii) The same protocol as described in (i), but Hb (10 μM) was applied before application of caffeine (indicated by arrow). (b) After depletion of Ca^{2+} stored in smooth muscle cells as in (a), Ca^{2+} (2.6 mM) was applied with or without Hb for various periods (X min), then 3 min after return to Ca^{2+}-free solution, caffeine (20 mM) was applied. The grouped data are all shown in graph below. The contraction amplitude evoked by 10 min application of Ca^{2+} was normalized as a relative tension of 1.0: (○) control and (▲) in the presence of Hb (10 μM).

by the first application of caffeine. When Hb (10 μM) was applied 5 min before and during applications of caffeine (2 mM) in the above experiments, the amplitude of the contractions evoked by 2 or 20 mM remained the same as those observed before application of Hb [Figure 4a, (i) vs (ii)].

Figure 4b shows the effects of Hb on caffeine-induced contraction of intact muscle tissues. The tissue was perfused with Ca^{2+}-free solution and caffeine (20 mM) was repetitively applied to deplete the Ca^{2+} stored in the cells, then Ca^{2+} (2.6 mM) was applied for various periods (up to 10 min) in the presence or absence of Hb (10 μM). After a subsequent period of 3 min in Ca^{2+}-free solution, caffeine (20 mM) was applied so as to allow an estimation of the amount of Ca^{2+} stored in the sarcoplasmic reticulum. Amplitudes of the caffeine-induced contraction increased in a time-dependent fashion and reached a steady level 3 min after application of Ca^{2+}. When Hb (10 μM) was added to the Ca^{2+} containing solution, the contraction amplitude provoked by 1 min after application was consistently enhanced.

Effects of Hb on the Ca^{2+}-induced contraction in skinned muscle tissues

In skinned muscle tissues, the minimum concentration of Ca^{2+} required to produce a contraction was 0.1 μM, and the maximum response was evoked by 10 μM Ca^{2+}. The maximum amplitude of the contraction induced by STA_2 (10 nM) or K^+ (128 nM) was 50–70% of that induced by Ca^{2+} (10 μM). These observations confirm the previous observations made on these tissues (Nishiye *et al.*, 1988). After the contraction had reached a steady amplitude following cumulative accumulation of stepwise increase in Ca^{2+}, Hb (10 μM) had no effect on the contraction evoked by any given concentrations of Ca^{2+} (not shown).

Prevention of the EDRF-induced relaxation by Hb in the guinea-pig basilar artery

To observe the effects of Hb on the ACh-induced relaxation of intact tissues that were contracted by STA_2, ACh (10 μM) was applied for 15 min. As shown in Figure 5a, ACh (10 μM) relaxed the precontracted tissue by STA_2 (0.3 nM or 3 nM) nearly completely and the relaxation was maintained over 15 min with only a slight restoration in the contraction amplitude. When Hb (10 μM) was applied 10 min before and during application of STA_2, ACh transiently relaxed the tissues to a level close to the resting level but the contraction re-developed. Consistently, the amplitude of the contraction and the initial relaxation induced by ACh (10 μM) evoked by STA_2 (3 nM) and STA_2 (0.3 nM) were the same in the presence as in the absence of Hb (10 μM), but the relaxation was gradually attenuated in the presence of Hb. Figure 6 summarises the effects of Hb on the ACh-induced relaxation of tissues that were precontracted by STA_2 against time.

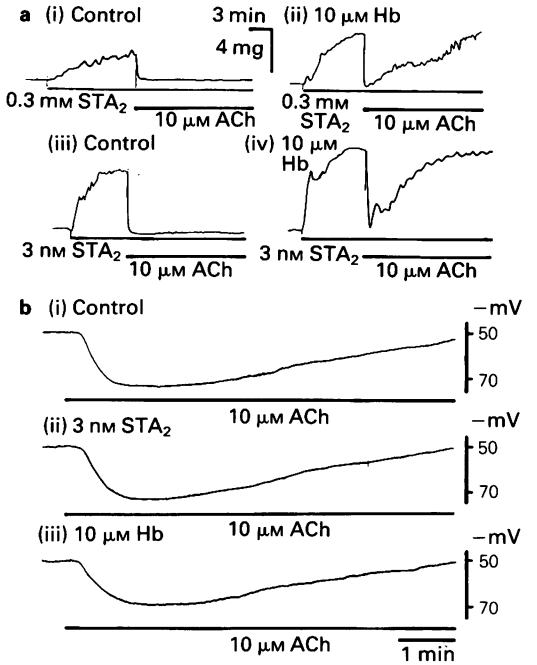

Figure 5 Effects of oxyhaemoglobin (Hb, 10 μM) on the time courses of relaxation of tissues that were precontracted by STA_2 (a) and on hyperpolarization induced by acetylcholine (ACh, 10 μM) in the presence or absence of STA_2 (3 nM) (b). (a) Relaxations were induced by ACh (10 μM) following application of STA_2 (0.3 nM and 3 nM) in the presence (ii) and (iv) or absence of Hb (10 μM) (i) and (iii). Hb was applied 10 min before application of STA_2. (b) Amplitudes and time courses of the hyperpolarization induced by ACh (10 μM) in the presence or absence of Hb. (i) effect of ACh (10 μM); (ii) effect of ACh following application of STA_2 (3 nM); (iii) effect of Hb (10 μM) on the ACh-induced hyperpolarization following applications of STA_2. STA_2 and Hb were applied 10 min before application of ACh.

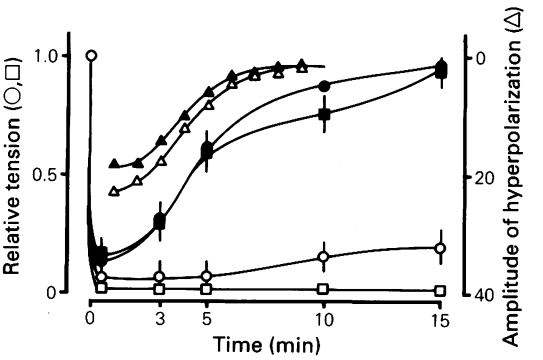

Figure 6 Time courses of the relaxation and hyperpolarization induced by acetylcholine (ACh) in the presence or absence of oxyhaemoglobin (Hb, 10 μM). Left vertical axis indicates the relative tension (the amplitude of the contraction evoked by STA_2 (0.3 or 3 nM) in the presence or absence of Hb were normalized as 1.0, respectively). Right vertical axis indicates the membrane potential (indicated by the absolute value). The STA_2-induced contractions (0.3 nM and 3 nM) in absence of Hb (○, □), in the presence of Hb (●, ■); (△, ▲) membrane potential measured before and after application of Hb. ACh was applied just after 0 min.

Effects of Hb on the ACh-induced changes in membrane potential in smooth muscle cells of the guinea-pig basilar artery

The resting membrane potential of smooth muscle cells of the guinea-pig basilar artery was -50.8 ± 1.2 mV ($n = 30$, 3 preparations) and this value was much the same as that measured in other investigations (guinea-pig: Fujiwara & Kuriyama, 1983; 1984; dog: Fujiwara *et al.*, 1982). The smooth muscle cells were electrically quiescent and spontaneous discharges were not obtained.

Application of ACh (10 μM) caused marked hyperpolarization of the membrane (the peak amplitude was 18 mV, $n = 5$) and this gradually attenuated over several minutes (Figure 5b(i)). When STA_2 (3 nM) was applied, there was no change in membrane potential and subsequent application of ACh (10 μM) hyperpolarized the membrane to the same extent as observed on application of ACh alone (Figure 5b(ii)). Pretreatment with Hb (10 μM) did not modify the membrane potential but did slightly reduce the peak amplitude of the hyperpolarization induced by ACh (10 μM) in the presence of STA_2 (at 3 nM; 15 mV, $n = 5$), without affecting the duration of the hyperpolarization (Figure 5b(iii)). In endothelium-denuded tissue, ACh (10 μM) did not produce hyperpolarization. In Figure 6, the time course of hyperpolarization induced by ACh in the presence or absence of Hb can be compared with the relaxation induced by the same procedures. As can be seen, the time course of the hyperpolarization observed both in the presence and in the absence of Hb coincided well with the time course of the relaxation induced by ACh in the presence of Hb.

A23187 (0.01–1 μM) relaxed the STA_2-contracted tissues in a concentration-dependent fashion (Figure 7a). A23187 (0.01–0.1 μM) did not modify the membrane potential but at a concentration of 1 μM, A23187 caused a slight though statistically insignificant hyperpolarization. The effects of A23187 on the STA_2-induced contraction and membrane potential are summarized in Figure 7b.

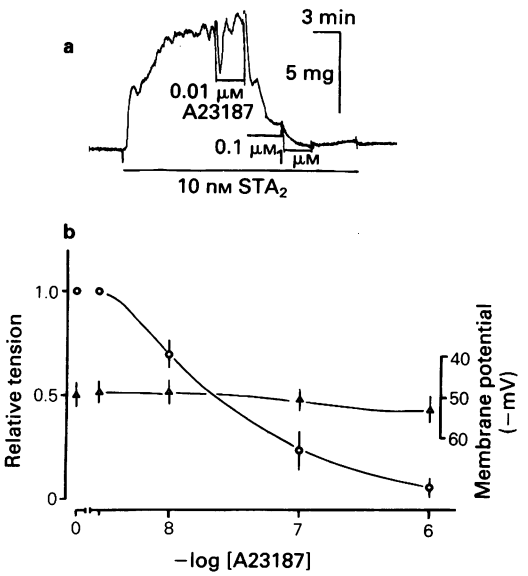

Figure 7 The effects of A23187 on the STA_2-induced contraction (a) and on the membrane potential and mechanical response (b). (a) A23187 was cumulatively applied during the contraction evoked by STA_2 (10 nM). (b) Effects of various concentrations of A23187 on the membrane potential (△, $n = 10$–15; 3 preparations) and mechanical responses (○, $n = 5$). (The STA_2-induced contraction before application of A23187 was normalized as 1.0.)

Discussion

The present study shows that in the guinea-pig intact basilar artery, ACh relaxes preparations that were precontracted by STA_2 and suggest that this is caused by release of EDRF and an endothelial-dependent hyperpolarizing factor (EDHF). It is possible that the latter, like EDRF, is released from the endothelium. As estimated from the actions of Hb and the time course of hyperpolarization, it seems that EDRF produced the long lasting relaxation and the EDHF contributed to an initial transient relaxation.

The thromboxane A_2 (TXA_2) analogue, STA_2, produced a larger contraction amplitude in this tissue in comparison to that induced by ACh, 5-hydroxytryptamine or other agonists (Nishiye *et al.*, 1988). This contrasts with the rabbit coronary artery, in which the maximum contractile amplitude induced by ACh was larger than that evoked by STA_2 (100 nM). Kanmura *et al.* (1987) deduced that the STA_2-induced contraction was due to activation of receptor-operated Ca^{2+} influx and also to intracellular release of Ca^{2+} from the sarcoplasmic reticulum following activation of the TXA_2 receptor. The present results on the nature of contraction evoked in Ca^{2+}-free solution containing EGTA support that conclusion. In the presence of a Ca antagonist (nifedipine), the amplitude of the contraction evoked by STA_2 was reduced but Hb partially restored it. Therefore, the release of EDRF may not be but the Ca^{2+} mobilisation in smooth muscle may be inibited by nifedipine. However, the underlying mechanism of restoration of the nifedipine-induced inhibition by Hb has not been resolved in the present experiments.

In intact tissues, Hb enhanced the amplitude of the contraction produced by high K^+ or by STA_2. The tonic component of the K^+-induced contraction was enhanced to a greater extent by Hb than the phasic, but both components of responses induced by STA_2 were enhanced by Hb. Hb did not modify the release of Ca^{2+} from the intracellular stores induced by application of STA_2 or by caffeine in Ca^{2+}-free solution containing EGTA but Hb did enhance the accumulation of Ca^{2+}, as estimated from the amplitude of the caffeine-induced contraction amplitude. Since Hb had no effect on the Ca^{2+}-induced contraction in skinned muscle tissues, it may be that Hb can inhibit the action of EDRF released spontaneously or by stimulation with an agonist. Thus enhancement of the amplitude of the contraction probably occurs by increasing the amount of free Ca^{2+} in the cytosol rather than by direct actions on the contractile proteins.

It is known that Hb inhibits the relaxation of precontracted tissue caused by release of EDRF, via inhibition of guanylate cyclase in the cytosol (soluble guanylate cyclase) using the same mechanism as the inhibition of relaxation induced by nitro-compounds (Miki *et al.*, 1977; Murad *et al.*, 1978; Mittal *et al.*, 1978; Gruetter *et al.*, 1979; 1981; Itoh *et al.*, 1985). In fact, EDRF-induced relaxation is thought to be related to synthesis of cyclic GMP in the cytosol through a nitric oxide residue of EDRF (Furchgott & Jothianandan, 1983; Palmer *et al.*, 1987). Nitroglycerin and nicorandil apparently reduce the amount of free Ca^{2+} in the cytosol, judging from experiments with the fluorescent indicator quin2 (Sumimoto *et al.*, 1987). Moreover, cyclic GMP is known to accelerate the Ca pump at the sarcolemma (Suematsu *et al.*, 1984; Itoh *et al.*, 1985; Popescu *et al.*, 1985; Sumimoto *et al.*, 1987). Since Hb enhanced the amplitude of the contraction evoked by STA_2 to a greater extent than the contraction induced by high K^+, STA_2 may cause more release of EDRF than high K^+. However, the tonic component of the high K^+-induced contraction was markedly enhanced by Hb, so the possibility that high K^+ itself induces EDRF release cannot be ruled out. These findings are consistent with previously

published data obtained with the dog coronary artery (Robertson, 1985). Furthermore, it is also apparent from the present results that in endothelium-denuded tissue, the amplitude of the tonic contraction was of much the same amplitude as the phasic component. Thus the relatively low amplitude of tonic component in comparison to the phasic component of the K^+-induced contraction in intact tissues could be partly explained by release of EDRF by high K^+.

Hb inhibited the ACh-induced relaxation of tissues that were precontracted by STA_2, such that the ACh-induced relaxation was sustained for over 15 min with only a gradual recovery. However, in the presence of Hb, ACh caused transient relaxation. This indicates that Hb does not completely prevent the endothelium-dependent relaxation.

Komori & Suzuki (1987a,b) found that in the rabbit saphenous artery, stimulation of endothelium by ACh releases EDRF and EDHF. The hyperpolarization induced by ACh was transient and ceased within several minutes. Such hyperpolarization and sustained relaxation also occurred in the pig coronary artery (Beny *et al.*, 1986) and in the rat aorta (Southerton *et al.*, 1987). Furthermore, Komori & Suzuki (1987a) observed that although both ACh and oxotremorine cause release of EDRF and relax the precontracted rabbit saphenous artery, only ACh hyperpolarizes the membrane. Similarly Bolton & Clapp (1986) reported that substance P produces relaxation of guinea-pig mesenteric artery that had been precontracted by noradrenaline without inducing any hyperpolarization of the membrane. Thus, ACh seems to release EDRF and EDHF from the endothelium in both tissues but by independent mechanisms. The present results obtained from the guinea-pig basilar artery suggest that ACh causes relaxation by releasing both EDRF and EDHF; the initial relaxation is mainly due to the hyperpolarization of the membrane as observed in the presence of Hb and the sustained relaxation is mainly due to release of EDRF. It should however be noted that the electrical and mechanical activities of the basilar artery in response to ACh were recorded at different temperatures.

The Ca ionophore, A23187 is also known to be a stimulant of EDRF release (Rapoport & Murad, 1983; Griffith *et al.*, 1984a,b; Cocks *et al.*, 1985) and in fact, A23187 relaxed the basilar artery which had been precontracted by STA_2. However, A23187 did not produce hyperpolarization of the membrane. This is surprising since release of EDRF has been reported to be Ca-dependent (Winquist *et al.*, 1985), moreover, we found that nifedipine did not prevent EDRF-induced relaxation. These observations suggest that the cellular mechanisms responsible for release of EDRF and EDHF differ (Komori & Suzuki, 1987b) and also that unlike EDRF, EDHF release cannot be evoked by the Ca^{2+} ionophore.

Fujiwara & Kuriyama (1983, 1984) reported that in the guinea-pig basilar artery haemolysate caused depolarization of the membrane which was associated with an increase in membrane resistance. Presumably haemolysate reduced the K^+ conductance of the membrane. Since we have now found that Hb does not depolarize the membrane in this tissue, it seems reasonable to postulate that factors in the haemolysate other than Hb may have contributed to the depolarization.

In the basilar artery, multiple substances may be released during activation of endothelial cells, not only EDRF and EDHF but also endothelium-derived contracting factor (De Mey & Vanhoutte, 1983; Nagao & Suzuki, 1987) and also relaxing substances, such as prostacyclin (PGI_2) (Moncada *et al.*, 1977; Baenziger *et al.*, 1977; Eldor *et al.*, 1981; Domae & Kuriyama, 1986). It is unlikely that the effects of ACh on the guinea-pig basilar artery can be accounted for by the release of PGI_2 since the relaxant response to ACh is unchanged by indomethacin and PGI_2 does not hyperpolarize the membrane (unpublished observations). Most of these substances except for endothelium-derived contracting factor tend to produce relaxation of vascular tissues. Cerebral vasospasm, occurring following subarachnoid haemorrhage, may be partly caused by synthesis of thromboxane A_2 or leukotriene D_4 (Tagari *et al.*, 1983; Rosenblum, 1985; Busija *et al.*, 1986) by the platelets, leucocytes and erythrocytes contained within the clot (coagulate). Thus, Hb contained in the clot may act as a stimulant of vascular smooth muscle cells and also as an inhibitor of EDRF-induced relaxation, so causing vasoconstriction. Thus, if endothelial cells were damaged following subarachnoid haemorrhage, vasoconstriction may well be accelerated.

The present results demonstrate that EDRF and EDHF may be released from the endothelium in the guinea-pig basilar artery via different mechanisms and thus may contribute to the maintenance of low resting muscle tone. Cerebral vasospasm provoked following subarachnoid haemorrhage may in part be caused by damage of endothelium and/or by substances released from the clot, such as Hb (Fisher *et al.*, 1980; Ohta *et al.*, 1980).

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