Role of Endothelium in Responses of Vascular Smooth Muscle

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A FEW years ago, we discovered that the relaxation by acetylcholine (ACh) of isolated preparations of arteries precontracted by addition of an exogenous stimulating agent (e.g., norepinephrine) is strictly dependent on the presence of endothelial cells (Furchgott and Zawadzki, 1980a, 1980b; Furchgott et al., 1981; Furchgott, 1981). Our initial study was on the descending thoracic aorta of the rabbit (henceforth referred to as rabbit aorta), but it was soon found that the requirement for endothelial cells for relaxation by ACh applied in the case of isolated arteries from all mammalian species tested. Using rabbit aorta, we demonstrated that this endothelium-dependent relaxation by ACh is initiated by an action of this agent on a muscarinic receptor of the endothelial cells. We also successfully demonstrated that the action of ACh on this receptor stimulates the endothelial cells to release some factor (or factors) which then acts on the smooth muscle cells of the artery to cause them to relax (Furchgott and Zawadzki, 1980b; Furchgott et al., 1981).

Since our first work on ACh, we and others have found additional agents that require the presence of endothelial cells to produce all or part of their relaxation of isolated preparations of arteries. Among these agents are the calcium ionophore A23187 (Zawadzki et al., 1980; Furchgott, 1981; Furchgott et al., 1983); ATP and ADP (De Mey and Vanhoutte, 1980, 1981; Furchgott and Zawadzki, 1980c; Furchgott, 1981); substance P (Zawadzki et al., 1981); bradykinin, in the case of canine and human arteries (Cherry et al., 1981, 1982; Chand and Altura, 1981b, Altura and Chand, 1981); histamine, in the case of the rat aorta (Van de Voorde and Leusen, 1983); and thrombin, in the case of canine arteries (De Mey and Vanhoutte, 1982; De Mey et al., 1982; Ku, 1982).

Prior to our finding of the obligatory role of endothelial cells for relaxation of arteries by ACh, others had shown that endothelial cells, either present on the intimal surface of blood vessels or in culture, can produce prostaglandins, including prostacyclin (PGI2) (Moncada et al., 1977; McIntyre et al., 1978; Weksler et al., 1977). Also, it was already known that endothelial cells cultured from human umbilical veins could be stimulated to synthesize PGI2 by thrombin, trypsin, and the ionophore A23187 (Weksler et al., 1978), and that PGI2 was a potent vasodilator of vascular beds and a relaxant of many isolated arteries (Moncada et al., 1978, 1979; Needleman et al., 1978). In view of the existing information about prostaglandins, the possibility was considered that PGI2 or some other prostaglandin was the endothelium-derived relaxing factor (EDRF) mediating the ACh-induced relaxation of the isolated rabbit aorta. However, this possibility was soon ruled out when it was found that none of the prostaglandins tested (PGI2, PGE2, PGE1, PGA2, PGF2a) had any relaxing effect on rabbit aorta (although some gave contractions), and that cyclooxygenase inhibitors (indomethacin and aspirin) did not inhibit the ACh-induced relaxation (Furchgott and Zawadzki, 1980a, 1980b).

The present review will deal with the following topics: the characteristics of the relaxation of isolated arteries by ACh and the evidence for its mediation by EDRF, agents and conditions that interfere with the relaxation by ACh, the role of calcium in the release of EDRF, other agents that relax arteries in an endothelium-dependent manner, the role of endothelial cells in the relaxation of veins and peripheral resistance vessels, proposed roles for endothelial cells in facilitating contractions of blood vessels, and speculation about the nature of EDRF and the mechanism by which it produces relaxation.

I. Characteristics of Relaxation of Arteries by ACh

A. Requirement for Endothelial Cells

Figure 1 is a typical record showing the effect of rubbing the intimal surface of a ring of rabbit aorta on response of the preparation to ACh. In the un-
rubbed preparation, precontracted to a moderate level of tone, ACh produces graded relaxation at low concentrations, followed by some contraction at higher concentrations, but in the rubbed preparation, ACh produces no relaxation. The loss of the relaxing response to ACh in aortic preparations (rings and helical and transverse strips) that had been either intentionally rubbed on the intimal surface or unintentionally rubbed (as must have been the usual case during the preparation of helical strips in earlier studies) suggested to us that endothelial cells, which might well be removed by gentle rubbing, are necessary for the production of relaxation by ACh (Furchgott and Zawadzki, 1980b). The strict dependence of the relaxation on the endothelial cells was then established when both light microscopy, employing an en face silver-staining method, and scanning electron microscopy (Furchgott and Zawadzki, 1980b; Furchgott et al., 1981) showed that complete removal of endothelial cells from the intimal surface of the rabbit aorta, either mechanically by rubbing or enzymatically by collagenase, resulted in complete loss of the relaxing response to ACh. In carefully prepared rings of aorta that showed excellent relaxation to ACh (as in Fig. 1), there was usually about 70–80% of the intimal surface still covered by endothelium on histological examination at the end of an experiment. Histological evidence for the obligatory role of endothelial cells in the ACh-induced relaxation has also been obtained in our laboratory and other laboratories for selected arteries from the dog and cat, as well as from the rabbit (unpublished observations; De Mey and Vanhoutte, 1981, 1982; Singer and Peach, 1982; Lee, 1982). Our early work showed that the relaxations by a wide variety of agents, including isoproterenol, sodium nitrite, glycercyl trinitrate, azide, adenosine, and AMP, are not influenced by removal of endothelial cells (Furchgott and Zawadzki, 1980b). In addition, relaxation of arteries by papaverine and relaxation by prostaglandins such as PGI$_2$ and PGE$_2$ are not dependent on the presence of endothelial cells (unpublished observations; Chand and Altura, 1981b; Lee, 1982; Cherry et al., 1982; De Mey et al., 1982). Also, reversible relaxation of rabbit aorta during irradiation with long wave ultraviolet light (Furchgott et al., 1961) does not require the presence of endothelial cells (Furchgott and Zawadzki, 1980b).

Very early, it was demonstrated that the endothelial cell receptor on which ACh acts is of the muscarinic type (Furchgott and Zawadzki, 1980b; Furchgott et al., 1981). This was shown in the case of the rabbit aorta by the high potency of atropine as a competitive blocking agent (apparent $K_B$ of 0.35 nm), as well as by the relative potencies of the muscarinic agonists ($ACh >$ methacholine > carbachol). As was to be expected, atropine also effectively blocked relaxation by ACh in arteries of all species tested (unpublished observations; De Mey and Vanhoutte, 1981; Chand and Altura, 1981b; Lee, 1982; Gordon and Martin, 1983). The contraction elicited by higher concentrations of ACh on rabbit aorta (see Fig. 1) and some other arterial preparations is also the result of an action on muscarinic receptors, but these receptors mediating contraction are on the smooth muscle cells (Furchgott and Zawadzki, 1980b; Furchgott et al., 1981; De Mey and Vanhoutte, 1981). [It should be noted, also, that, in some arteries, ACh at very high concentrations (≥0.1 mm) will cause transient contractions by stimulating nicotinic receptors in adrenergic nerve terminals, so that the terminals release norepinephrine which mediates contraction (Steinsland and Furchgott, 1975).] Whether the muscarinic receptor on the endothelial cells is identical to that on the smooth muscle cells is not yet known. In a number of experiments with rabbit arteries, pilocarpine, which acts as a partial agonist on many muscarinic receptor-effector systems, failed to produce relaxation, but acted as a competitive antagonist against relaxation by ACh (Furchgott et al., 1981).

B. Influence of the Degree of Contraction and Nature of the Contracting Agent

In most experiments on rabbit aorta, NE has been used to produce a tonic contraction on which ACh has then been tested for its relaxing activity (Furchgott and Zawadzki, 1980b; Furchgott et al., 1981). Against moderate NE-induced tone (about 25–50% of maximal contraction with NE), ACh at 1 μM usually is able to produce 90–100% relaxation. Half-maximal relaxation usually occurs between 0.03 and 0.1 μM ACh. In the face of maximal contraction...
produced by NE (30 \mu M), ACh can usually produce about 30% relaxation. Moderate contractions of rabbit aorta produced by histamine, serotonin, angiotensin II, or PGE_2, have essentially the same sensitivity to the relaxing effect of ACh as do those produced by NE (Furchgott et al., 1981).

In our studies on other isolated arteries from the rabbit (celiac, superior mesenteric, ear, renal), the cat (thoracic and abdominal aorta, superior mesenteric, renal, pulmonary, external iliac), and the dog (femoral, superior mesenteric, inferior mesenteric, celiac, renal, pulmonary, and circumflex and left anterior descending coronary), we have found their sensitivities to the relaxing action of ACh to be about the same or greater than that of the rabbit aorta. In a limited number of human arteries (branches of mesenteric arteries and an ovarian artery), sensitivity to the relaxing action of ACh has been less than that in the arteries from laboratory animals (unpublished observations). Other investigators also have reported on the high sensitivity of certain isolated dog arteries to ACh (De Mey and Vanhoutte, 1981; De Mey et al., 1982; Chand and Altura, 1981b; Altura and Chand, 1981).

In rabbit aorta, moderate contractions produced by increasing K^+ in the Krebs solution (to 18–24 mM) are less sensitive to ACh than are equivalent contractions produced by NE, with the maximum relaxation by ACh of K^+-contractions being only 10–50% of that of NE contractions (Furchgott et al., 1981). In a few experiments, we also found that even the strong contractions of rabbit aorta produced by bathing the preparation in a completely depolarizing \(K_2SO_4\)-Krebs solution (all NaCl and NaHCO_3 of the regular solution replaced by \(K_2SO_4\) and KHCO_3) could be relaxed about 20% by 1 \mu M ACh. De Mey and co-workers (1980b, 1982), using rings of dog femoral artery, also found that the contractions produced by elevated K^+ were less sensitive to the relaxing action of ACh than were contractions produced by NE. Chand and Altura (1981b) compared the relaxations by ACh of spiral strips of canine intrapulmonary arteries contracted by 80 mM K^+ and by NE, and reported that the relaxation in the face of the K^+-induced tone was only 5% of that in the face of NE-induced tone ("95% inhibition"). However, in five experiments on rings of these arteries in our laboratory, tested at equivalent levels of contraction produced by 80 mM K^+ and by NE, ACh-induced relaxation in the face of the K^+-induced tone ranged from 10 to 40% of that in the face of NE-induced tone. Thus, sensitivity to ACh of these dog arteries was similar to that of rabbit aorta when contracted with excess K^+.

C. Demonstration of Release of an Endothelium-Derived Relaxing Factor (EDRF)

Evidence that a relaxing factor (or factors) is produced and released by endothelial cells when stimulated by ACh was first obtained with rabbit aortic strips, with a procedure involving the suspension of pairs of strips together in a so-called "sandwich" mount (Furchgott and Zawadzki, 1980b; Furchgott et al., 1981). In this procedure, a transverse strip freed of endothelial cells was tested when mounted separately and also when mounted, intimal surface against intimal surface, with a longitudinal strip of the same width and length with endothelial cells present ("sandwich mount"). Because the muscle cells of the longitudinal strips were oriented at a right angle to the connection to the force transducer, this strip could contribute only trivial changes in tension on the transducer during its contraction. ACh, which failed to relax the contraction of the endothelium-free transverse strip mounted separately, always gave good relaxation of the same strip when it was mounted in a sandwich with the endothelium-containing longitudinal strip. Thus, the endothelial cells of the latter strip in response to ACh must have released some factor(s) which, on diffusion into the transverse strip, activated relaxation of the contracted muscle cells of the latter. With this procedure, we have also demonstrated release of an EDRF by the ionophore A23187 in the case of rabbit aortic strips. (It should be noted that we have also had occasional success in demonstrating release of EDRF in superfusion experiments in which ACh is added to the superfusion fluid that first passes over the endothelium of an intact segment of aorta and then over a suspended transverse strip free of endothelium. However, the results with superfusion procedures have been discouragingly inconsistent up to this time.)

Van de Voorde and Leusen (1983) have reported successful application of a modified sandwich method in demonstrating release of EDRF in response to both ACh and histamine in the case of strips of rat aorta. Also, the sandwich method or modifications of it have been applied successfully in our laboratory and in others to show release of EDRF in other arteries of rabbits and dogs. In modifications developed independently by J. De Mey and by G.W. Frank and J. Bevan (personal communications), a ring of artery without endothelium is mounted for recording with either a strip of artery with endothelium, or a smaller inverted ring with endothelium, inserted in its lumen.

Another modification of the sandwich method was developed in our laboratory to demonstrate release of EDRF in the case of the dog intrapulmonary arteries, after Chand and Altura (1981b) had expressed doubts about the role of released EDRF in the relaxation of those arteries by ACh and by bradykinin. The original method is not suitable in the case of these arteries because their muscle cells are not all arranged in an essentially circular pattern (as in the rabbit aorta), and, therefore, longitudinally cut strips can also exert appreciable tension changes on the force transducer when contracted by NE. In order to keep the strip containing endothelial cells (donor) from contracting in response to NE when it is mounted in a sandwich with the strip freed of
endothelial cells (recipient), the donor strip is pre-treated separately with sufficient dibenamine to block irreversibly and completely the α-adrenergic receptors of the smooth muscle. Using this procedure, we have demonstrated release of EDRF by both ACh and bradykinin in the case of dog intrapulmonary arteries (Fig. 2).

II. Agents and Conditions that Interfere with the Endothelium-Dependent Relaxation of Arteries by ACh

A. Anoxia

Prior to the discovery that relaxation of arteries by ACh requires the presence of endothelial cells, De Mey and Vanhoutte (1978, 1980) had found that anoxic conditions inhibited the relaxation by ACh of rings of canine femoral arteries. We showed that a lack of oxygen also inhibited relaxation of rings of rabbit thoracic aorta by ACh (Furchgott and Zawadzki, 1980; Furchgott et al., 1981). To obtain consistent complete inhibition, we had to use a specially prepared 95% N2-5% CO2 gas mixture (less than 0.001% O2 contamination) and an experimental arrangement to minimize any leakage of air into the muscle chamber during gassing with the mixture and adding of drugs. The same anoxic condition did not interfere with relaxation by NaN02, glyceryl trinitrate, and isoproterenol. A reduction of O2 in the gassing mixture from 95% to about 5% decreased the relaxation response to ACh moderately in isolated canine femoral arteries (De Mey and Vanhoutte, 1980), but did not significantly change it in isolated canine intrapulmonary arteries (Chand and Altura, 1981b).

B. 5,8,11,14-Eicosatetraynoic Acid (ETYA)

As pointed out in the introduction, inhibition of cyclooxygenase does not interfere with the relaxing effect of ACh on rabbit aorta and other arteries. However, we did find that ETYA, the triple-bond analog of arachidonic acid, which is recognized as a lipoxygenase inhibitor as well as a cyclooxygenase inhibitor (Flower, 1974), was an effective inhibitor of relaxation by ACh. When ETYA is added during the course of an ACh-induced relaxation of rabbit aorta that has been precontracted with NE, it rapidly (in less than 1 minute) antagonizes the relaxation (i.e., it restores contraction) (Furchgott and Zawadzki, 1980).

Figure 2. "Sandwich" experiment demonstrating that bradykinin (BKN) releases a nonprostaglandin relaxing factor from endothelial cells of canine intrapulmonary artery. Transverse strips were 2.5 mm wide and about 12 mm long. Small plastic clips were used for attachments in mounting the strips. Flurbiprofen (35 μM) was present in Krebs solution to prevent prostaglandin synthesis. (1) Strip A, with endothelial cells present, was relaxed by BKN, but strip B, with endothelial cells mechanically removed by rubbing of the intimal surface, was not relaxed but was further contracted by BKN. (2) Strip A, after exposure to 10 μM dibenamine for 30 minutes (to irreversibly block α-adrenergic receptors) and washout, no longer was contracted by NE, even at very high concentrations. (3) Strip A and strip B were now mounted together in a "sandwich" arrangement, with their intimal surfaces apposed, in a single pair of clips, and then were reequilibrated for 30 min. Strip B in the sandwich, after contraction by NE, was now relaxed when BKN was added. Since precontracted strip B alone was not relaxed by BKN, its relaxation in the sandwich must have been evoked by relaxing factor released from the endothelial cells of strip A by BKN. (4) Final testing confirmed that strip B alone still was not relaxed by BKN, and that strip A alone still was not contracted by NE. Contraction of strip A with angiotensin II (ANG) allowed endothelium-dependent relaxation by BKN to be demonstrated once more.
wadzki, 1980b; Furchgott et al., 1981). The antagonism is concentration-dependent and is almost complete at 100 μM ETYA (Fig. 3). This agent does not antagonize relaxations of rabbit aorta produced by isoproterenol, NaNO₂, glyceryl trinitrate, adenosine, or adenylic acid (AMP), and by itself it inhibits to some extent contractions evoked by NE and other contracting agents. On the rabbit superior mesenteric artery, cat superior mesenteric artery, and a variety of dog arteries, the degree of acute antagonism of ACHe-induced relaxation by ETYA is generally less than on the rabbit aorta (unpublished observations; Cherry et al., 1981), possibly because of a greater inhibitory effect of ETYA itself on contractions in these vessels. Pre-addition of ETYA (100 μM) significantly inhibits ACHe-induced relaxation in the isolated dog femoral artery (De Mey et al., 1982) and rat thoracic aorta (Van de Voorde and Leusen, 1983). In the case of rabbit aorta, the antagonistic action of ETYA is reversible on washout if the exposure time to the ETYA is limited to a few minutes. However, when aortic preparations are exposed to 100 μM ETYA for 30–60 minutes, they completely and irreversibly lose the capacity to relax in response to ACHe (Furchgott and Wadzki, 1980b; Furchgott et al., 1981).

C. Quinacrine

Quinacrine (mepacrine), which is known to be an inhibitor of phospholipase A₂ (Flower and Blackwell, 1976), can inhibit ACHe-induced relaxation of rabbit thoracic aorta, whether it is added prior to or after the addition of the ACHe (Furchgott and Wadzki, 1980b; Furchgott et al., 1981; Singer and Peach, 1983b). The inhibition is essentially complete at concentrations of 10–30 μM, and is readily reversed by washout. As in the case of ETYA, the inhibition is selective, with quinacrine exhibiting no inhibition against relaxations produced by isoproterenol, NaNO₂, glyceryl trinitrate, and AMP. It has been reported that quinacrine (10 μM) partially inhibits in a noncompetitive manner contractions elicited by methacholine in rings of rabbit aorta devoid of endothelium (Singer and Peach, 1983b). Quinacrine has also been found effective in inhibiting the relaxing response to ACHe in some isolated canine arteries, such as the LAD coronary, the superior mesenteric (Furchgott et al., 1981; Cherry et al., 1982), and the femoral (De Mey et al., 1982). On the other hand, in rat thoracic aorta, quinacrine does not inhibit ACHe-induced relaxation at 10 μM (Van de Voorde and Leusen, 1983) and inhibits less than 40% at 100 μM (Rapoport and Murad, 1983). On some vessels, quinacrine itself, particularly at concentrations approaching 100 μM, tends to cause considerable inhibition of contractions by agents such as NE (unpublished observations; Singer and Peach, 1983b; Rapoport and Murad, 1983).

D. Nordihydroguaiaretic Acid (NDGA)

NDGA, which has two catechol moieties in its structure and is known to be both an antioxidant and an inhibitor of lipoxygenases, has also been found to be a very effective inhibitor of ACHe-induced relaxation in rabbit aorta (Furchgott et al., 1982, 1983; Singer and Peach, 1983b) and dog renal artery (Chand and Altura, 1981c). When it is added to aorta during the course of a relaxation by ACHe, it antagonizes the relaxation almost as rapidly as does an addition of ETYA (Furchgott et al., 1983). NDGA does not inhibit relaxations by endothelium-independent relaxing agents. The inhibition by NDGA of relaxation by ACHe is largely reversed on washout if exposure is limited to a few minutes. However, if exposure is prolonged (e.g., 30 minutes at 100 μM), essentially complete, irreversible inhibition results (Furchgott et al., 1982). On rabbit aortic rings precontracted with NE or PGF₂α, NDGA itself produces some moderate relaxation of tone (usually transient). Interestingly, this NDGA-induced relaxation itself requires the presence of endothelial cells (Furchgott et al., 1983). NDGA has also been found to rapidly antagonize (inhibit) relaxations by ACHe in the rabbit superior mesenteric artery and some dog arteries (unpublished observations), but these preparations are more sensitive to the relaxing effect of NDGA itself, and antagonism by NDGA to the relaxations by ACHe may therefore appear to be incomplete. In the case of the cat superior mesenteric artery, NDGA itself has such a marked relaxing effect (largely but not completely endothelium-dependent) that any...
antagonism by it of ACh-induced relaxations would be completely masked.

E. Hydroquinone

Fairly early in our work on the endothelium-dependent relaxation of rabbit aorta by ACh, we tested a number of potential free-radical scavengers as inhibitors, since one speculation at the time was that EDRF may be a free radical (Furchgott et al., 1981). Results with most of the agents tested were either negative or equivocal, but hydroquinone (100 µM), when added during the course of relaxation of rabbit aorta by ACh, antagonized the relaxation about as rapidly as did ETYA (Furchgott et al., 1981; Furchgott, 1981). Over-exposure to hydroquinone (100 µM for 10–20 minutes) completely and irreversibly eliminated relaxation by ACh (unpublished observations).

F. α-p-Dibromoacetophenone [p-Bromophenacyl Bromide (BPB)]

This alkylating agent, which has been shown to be a potent irreversible inhibitor of phospholipase A₂ in cell-free systems (Roberts et al., 1977), is a potent inhibitor of the relaxing action of ACh on rabbit aorta. An exposure to as little as 3 µM for 20 minutes or 10 µM for 5 minutes suffices to inhibit completely and irreversibly this relaxing action (Furchgott et al., 1982, 1983). A number of other arteries tested from the rabbit, dog, cat, and man all have shown loss of the relaxing response to ACh after exposure to BPB. It should be noted, however, that the complete inhibition of ACh-induced relaxation in rings of rabbit aorta after exposure to BPB is generally accompanied by loss of endothelial cells from the intimal surface, as judged by histological examination at the end of experiments, using the en face silver-staining method (unpublished observations). The loss of these cells may be partial or complete, but complete loss of the relaxing response after exposure to BPB can occur even when the loss of cells is partial.

G. Extracellular Monovalent Cations; Temperature; Ouabain

As already noted, the relaxation of arteries by ACh when they are precontracted by elevated extracellular K⁺ is significantly less than when they are precontracted by NE and other agents. De Mey and Vanhoutte (1980b) also found in the case of dog femoral artery precontracted with NE that the degree of relaxation by ACh was markedly decreased by replacing most of the Na⁺ in the Krebs solution with either Li⁺ or sucrose, and completely blocked after an hour of exposure of the artery to a K⁺-free solution. Also, with this artery, they found that exposure to ouabain (2–10 µM) for 15 minutes or reduction of temperature to 22°C markedly inhibited ACh-induced relaxation. Since the reductions in extracellular Na⁺ or K⁺, the exposure to ouabain, and the reduction of temperature, are all interventions that would inhibit Na⁺-K⁺-ATPase, De Mey and Vanhoutte felt that their results suggested a role for this enzyme in the relaxation produced by ACh.

III. Relaxation of Arteries by A23187 and the Role of Calcium in the Release of EDRF

The calcium ionophore A23187 was the next agent after the muscarinic agonists to be found to be dependent on endothelial cells for its relaxing effect on isolated rabbit aorta and other arteries (Zawadzki et al., 1980; Furchgott, 1981; Furchgott et al., 1983). This agent (added from ethanol solutions) is 10–30× more potent than ACh in producing relaxation of rabbit aorta (Fig. 4A). It is also more powerful: against high levels of contraction, the maximal relaxation by A23187 (0.1 µM) is always greater than that by ACh (1–3 µM). Relaxation by A23187, like that by ACh, is not inhibited by indomethacin or flurbiprofen (cyclooxygenase inhibitors). Also, the relaxation, like that by ACh, is reversibly inhibited by anoxia; reversibly inhibited by ETYA, hydroquinone or NDGA on short exposure, and irreversibly inhibited by each on prolonged exposure; and irreversibly inhibited by BPB. Release of an EDRF from endothelial cells of rabbit aorta by A23187 has also been demonstrated, using A23187 in place of ACh in the sandwich method (unpublished observations). Relaxation by A23187, unlike that by ACh, is not inhibited by quinacrine (Furchgott and Zawadzki, 1980; Singer and Peach, 1983b). Despite this lack of inhibition, our other findings provide strong evidence that A23187 and ACh stimulate release of the same EDRF. It has been postulated that quinacrine may interfere with relaxation by ACh, not by directly inhibiting some enzyme, such as phospholipase A, but by interfering with alterations in ion fluxes or Ca²⁺ coupling set off by an agonist acting on the muscarinic receptor of endothelial cells (Furchgott et al., 1981; Singer and Peach, 1983b). Such a mode of action by quinacrine could account for its lack of effectiveness against A2187-induced relaxation.

On many other arteries from rabbit, dog, cat, and man on which we have tested A23187, it is always more powerful than ACh as a relaxing agent. When a high dose of A23187 (1 µM) is added to a ring or strip of rabbit aorta for several minutes and then washed out, the relaxing action of the ionophore (manifested as a marked reduction in sensitivity to NE and other contracting agents) persists for long periods after the washout. Whenever A23187 is present at a concentration that produces maximal relaxation, and tone is restored by additional NE, ACh fails to produce any additional relaxation of the artery (Fig. 4B). It is proposed that this interference by A23187 with relaxation by ACh is attributable to A23187 fully activating the mechanism for production and release of EDRF in the endothelial cells, thus precluding any additional activation by...
ACh (Furchgott et al., 1983).

In part because of our findings with A23187, we hypothesized that an increase of calcium ions in the region of some key Ca++-activated enzyme (perhaps a phospholipase) might be an early step in the sequence of reactions mediating the release of EDRF by both ACh and A23187 (Furchgott et al., 1981). A23187, acting as an ionophore, and ACh, opening up a Ca++-channel coupled to muscarinic receptor activation, both might facilitate Ca++ influx into the region of the enzyme. A recent study by Singer and Peach (1982) has provided convincing evidence for a critical role of calcium. In their study they used A23187, methacholine (in place of ACh as the muscarinic agonist), and phynylephrine (as the contracting agent) on rabbit aortic rings with endothelium. They found that eliminating Ca++ from the incubation medium inhibited maximum methacholine-induced relaxations of approximately equivalent phenylephrine-induced contractions by 67%, and A23187-induced relaxations by 92%. They attributed the lesser degree of inhibition of the methacholine-induced relaxations to the possible utilization by methacholine of a “pool of Ca++ inaccessible to the ionophore.” They also found that the calcium channel blockers, verapamil and nifedipine, inhibited maximum methacholine-induced relaxation by about 40-45%, and maximum A23187-induced relaxation by about the same extent. The finding that the channel-blockers inhibited A23187-induced relaxation was unexpected (since the ionophore should transport Ca++ directly across cell membranes without the involvement of activated channels), and Singer and Peach speculated that it may reflect a direct interaction between the blockers and A23187.

IV. Other Agents that Produce Endothelium-Dependent Relaxations of Arteries

A. ATP and ADP

The finding that ATP and ADP exert most of their relaxing effects on isolated preparations of arteries indirectly through an action on endothelial cells was independently made by De Mey and Vanhoutte (1980, 1981), using rings of dog femoral artery, and by my colleagues and me, using rings of rabbit aorta (Furchgott and Zawadzki, 1980; Furchgott, 1981). In both of these preparations, concentration-dependent relaxations by ATP or ADP (1–100 μM) are markedly reduced after removal of endothelial cells, whereas the relaxations by AMP or adenosine (10–1000 μM) are the same before and after removal of endothelial cells. Gordon and Martin (1983) have made rather similar findings in the case of pig aorta, except that—in this artery—a major part of the relaxations by AMP and adenosine are also endothelium-dependent. On the dog artery, 30 μM theophylline abolishes relaxations by 500 μM adenosine without significantly affecting relaxations by 10 μM.
ATP (De Mey and Vanhoutte, 1981). On the rabbit aorta theophylline does not inhibit relaxation by adenosine or AMP, but in the presence of 1–3 mM adenosine or AMP (added in order to activate fully the purinergic receptors on the muscle cells mediating relaxation), ATP and ADP no longer produce any relaxation of preparations without endothelium, but still produce good relaxation of those with endothelium (Fig. 5). These findings indicate that, in dog femoral artery and rabbit aorta, ATP and ADP relax untreated arterial preparations containing endothelial cells by both a direct action on the muscle cell and a more powerful indirect action via the endothelial cells, whereas AMP and adenosine relax such preparations by a direct action on the muscle cells. It should be pointed out that the direct relaxing action of ATP and ADP on the muscle cells may actually be the result of an action of their metabolic products, AMP and/or adenosine. Pearson and Gordon (1979) have shown that cultured smooth muscle cells and endothelial cells from porcine aorta can readily degrade added ATP and ADP to AMP and adenosine.

In rabbit aorta, relaxations caused by ATP and ADP often are transient. Also in this preparation, with or without endothelial cells, ATP at a high concentration (1 mM) often elicits a transient contraction (Fig. 5). Endothelium-dependent relaxations by ATP are not inhibited by cyclooxygenase inhibitors in the rabbit aorta (Furchgott et al., 1983) or in the dog femoral artery (De Mey et al., 1982). ETYA and quinacrine added during endothelium-dependent relaxations of rabbit aorta by ATP, antagonize the relaxations, but not so completely as they antagonize the relaxations by ACh (Furchgott, 1981). On the other hand, ETYA and quinacrine have been reported to have no inhibitory action against relaxations of the dog femoral artery by ATP (De Mey et al., 1982). The reason for this difference is not clear.

B. Bradykinin on Canine, Porcine, and Human Arteries

In the first preliminary publication from our laboratory on the effects of bradykinin (BKN) on isolated arteries (Cherry et al., 1981), we reported that the relaxation of canine superior mesenteric and celiac arteries by this potent vasodilator at concentrations ranging from 1 to 1000 nM was strictly dependent on the presence of endothelial cells, and was not inhibited by indomethacin. In a preliminary publication appearing simultaneously with ours, Chand and Altura (1981a) also reported the requirement for endothelial cells in the relaxation by BKN of isolated canine pulmonary and renal arteries, but stated that indomethacin inhibited relaxant responses to BKN and that the threshold concentrations of BKN were $10^{-15}$ to $10^{-16}$ M. However, in later publications (Chand and Altura, 1981b; Altura and Chand, 1981), they revised their preliminary statements by reporting that indomethacin does not inhibit relaxation of these arteries by BKN and that threshold concentrations averaged around $10^{-11}$ to $10^{-10}$ M.

In a more detailed report from my laboratory, we noted that for each of a large variety of canine arteries (coronary, celiac, superior mesenteric, splenic, pulmonary, gastric, renal and femoral), relaxation by BKN occurred at threshold concentrations of 0.1–1 nM, was always lost after removal of endothelial cells, and was not inhibited by either of

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**Figure 5.** Demonstration of endothelium-dependent relaxation by ATP. Paired rings of rabbit aorta, with and without endothelial cells, were tested simultaneously. Initial testing showed greater sensitivity of the ring with endothelial cells to relaxation by ATP. AMP (3 mM) produced marked relaxation of NE-induced contraction in both rings (not shown). After higher concentrations of NE were added to restore contraction in the presence of the AMP, relaxation by ATP occurred only in the ring with endothelial cells. See text for further discussion.
two cyclooxygenase inhibitors, indomethacin and flurbiprofen (Cherry et al., 1982). We drew the conclusion that, in all canine arteries, BKN, like ACh, produces relaxation by stimulating release of a non-prostaglandin-relaxing factor from endothelial cells. In contrast to our finding of no interference by indomethacin, Toda (1977) found that this agent reduced (to a smaller extent the sensitivity of isolated canine coronary arteries to the relaxing action of BKN. We found that treatment of canine arteries with inhibitors of cyclooxygenase almost always gave considerable potentiation of the contracting activity of the agents used to produce initial tone (usually NE or PGF₂α). The potentiation presumably occurs because the isolated arteries, prior to treatment with the inhibitor, are synthesizing prostaglandins with relaxing activity (e.g., PGI₂ and PGE₁) that opposes the contracting activity of added NE or PGF₂α (Cherry et al., 1982). Our studies do not exclude the possibility that BKN stimulates the release of some prostaglandins (as well as EDRF) in canine arteries, but they do suggest that prostaglandins make little or no contribution to the relaxing effect of BKN. Recently, Gordon and Martin (1983) have reported that relaxation of the pig aorta by BKN is also endothelium-dependent and prostaglandin-independent.

In the absence of oxygen, BKN, like ACh, no longer relieves canine arteries (renal and pulmonary, unpublished observations). Exposure of canine arteries to BPB (10 μM for 5 minutes) irreversibly eliminates the relaxation response to BKN, just as it does to that of ACh. Also, in these arteries (including intrapulmonary), the relaxing response to BKN, like that to ACh, can be inhibited to some degree by quinacrine, ETYA, and NDGA, but not so completely as is the relaxing response to ACh in rabbit aorta (Cherry et al., 1982; Furchgott et al., 1982, 1983). Finally, in the presence of a concentration of A23187 (0.1 to 1 μM) sufficient to exert its maximal relaxing activity, BKN, like ACh, fails to give detectable relaxation in canine arteries. The similarity in the characteristics of the relaxation of canine arteries by BKN and by ACh is strong evidence that BKN and ACh release the same non-prostaglandin EDRF from the endothelial cells in these arteries.

Before our studies on BKN, there was evidence that it relaxes rabbit arteries indirectly by stimulating the release of a prostaglandin. It had been shown that inhibition of prostaglandin synthesis by indomethacin prevented BKN-induced relaxation of the isolated rabbit celiac artery (Aiken 1974) and BKN-induced vasodilation of the perfused rabbit mesenteric vasculature (Blumberg et al., 1977) and coronary vasculature (Needleman et al., 1975). We have confirmed Aiken’s findings on isolated preparations (rings) of rabbit celiac and superior mesenteric arteries, using either indomethacin or flurbiprofen as the cyclooxygenase inhibitor (Cherry et al., 1982). The prostaglandin mediating the relaxation produced by BKN is probably PGI₂ (Aiken, personal communication). Relaxation of isolated celiac and superior mesenteric arteries of the cat by BKN was also completely inhibited by the cyclooxygenase inhibitors. In the absence of cyclooxygenase inhibition, removal of the endothelial cells did not interfere with BKN-induced relaxation in the rabbit arteries; and usually, but not always, did not interfere in the cat arteries. Thus, it appears that other types of cells in these arteries, in addition to endothelial cells, are stimulated by BKN to release a relaxing prostaglandin(s) (Cherry et al., 1982). It is of considerable interest that BKN fails to relax isolated preparations of rabbit aorta (thoracic and abdominal) and renal arteries. These arteries, unlike the rabbit celiac and superior mesenteric arteries, also do not relax in response to PGI₂. Cat aorta and renal arteries also fail to relax in response to BKN, but here the renal arteries do relax in response to PGI₂ and PGE₁ (unpublished results).

We have tested BKN on rings of isolated human arteries (branches of mesenteric arteries and ovarian artery) in a few experiments (Cherry et al., 1982; Furchgott et al., 1983; unpublished observations). In all cases, relaxation was dependent on the presence of endothelial cells and was not reduced by inhibition of cyclooxygenase by indomethacin or flurbiprofen. Thus, in human arteries, as in canine, relaxation by BKN appears to be mediated by a non-prostaglandin EDRF.

C. Substance P

Relaxation of isolated arteries from rabbits, dogs and cats by substance P is strictly dependent on the presence of endothelial cells (Zawadzki et al., 1981; Furchgott et al., 1983). This peptide is the most potent endothelium-dependent relaxing agent studied in our laboratory, with threshold concentrations ranging from about 30 pM in rabbit aorta to 1 pM in dog celiac and superior mesenteric arteries. Despite its greater potency, substance P usually gives a smaller maximal relaxation than does ACh on the rabbit aorta—perhaps because desensitization to substance P (fade of the relaxation) occurs during the course of exposure. Desensitization to substance P occurs in all arteries on which it has been tested. Depending on the artery used, desensitization to a maximally effective dose (about 100 to 1000X threshold) is usually complete within 10 minutes. After full development of desensitization to substance P, there is no loss of sensitivity to the relaxing actions of ACh, A23187, and BKN (the last in the case of canine arteries). Thus, desensitization appears to be at the level of the receptor for substance P. Full sensitivity to substance P returns readily after washout of a desensitizing dose. Tests with various agents (e.g., cyclooxygenase inhibitors, ETYA, quinacrine, and A23187) indicate that relaxation by substance P has many of the same characteristics as relaxation by ACh.
Recently, we have found that kassanin, octa-cholecystokinin (CCK-8), physalaemin, and eledoisin also require endothelial cells to produce relaxations of isolated rabbit and dog arteries, and that cyclooxygenase inhibitors do not interfere with their relaxing actions (Zawadzki et al., 1983; unpublished observations.). Moreover, it was shown that these peptides fail to produce relaxation in the presence of a desensitizing dose of substance P—strongly suggesting that they cause relaxation by acting on the same set of receptors as substance P.

D. Histamine on Rat Thoracic Aorta

In tests in our laboratory, we found no relaxation by histamine of rings of rabbit aorta, but only constriction, whether or not endothelial cells were present. This constriction is inhibited by antagonists of histaminergic H₁-receptors. Recently, however, Van de Voorde and Leusen (1983) have reported that rings of rat thoracic aorta precontracted with NE exhibit dose-dependent relaxation to histamine (10–100 μM). According to them, this relaxation, like that produced by ACh (0.01–100 μM) in rat aorta, was dependent on the presence of endothelial cells, was not blocked by indomethacin, and was diminished significantly by hypoxia, ETYA, and hydroquinone. The relaxation by histamine was inhibited by mepyramine but not by cimetidine, indicating that histamine acts on an H₁-receptor of the endothelial cells of the rat aorta. Successful modified “sandwich” experiments demonstrating release of an EDRF from rat aortic endothelial cells by histamine were also reported.

E. Thrombin on Canine Arteries

De Mey et al. (1982) have recently demonstrated that bovine thrombin (0.1 to 10 U/ml) elicits a dose-dependent relaxation of precontracted rings of dog femoral artery; and that the relaxation, like that elicited by ACh, is lost when the rings have been denuded of endothelial cells. With successive tests over several hours the sensitivity of the preparation to thrombin decreased progressively, whereas sensitivity to ACh did not. Thrombin-induced relaxation was effectively inhibited by heparin. Thrombin had been previously shown to stimulate formation and release of PGI₂ in cultured endothelial cells (Weksler et al., 1978; Hong et al., 1980), but the possibility that relaxation by thrombin was mediated by PGI₂ was ruled out by the finding that inhibitors of cyclooxygenase and prostacyclin synthetase failed to inhibit the relaxation (De Mey et al., 1982). These investigators also found that two agents that inhibit relaxation by ACh, namely ETYA, and quinacrine (see Section II), failed to inhibit relaxation by thrombin. In a more recent paper, De Mey and Vanhoutte (1982) have shown that other canine arteries (splenic, pulmonary, saphenous) also exhibit an endothelium-dependent relaxation by thrombin. Also very recently, Ku (1982) has demonstrated endothelium-dependent relaxation of isolated canine coronary arteries by thrombin. Here too, relaxation was resistant to indomethacin and blocked by heparin. The relaxation response to each addition of thrombin was transient, giving way in time to a contractile response.

F. Arachidonic Acid

The inhibition of ACh-induced relaxation of rabbit aorta by anoxia, quinacrine, and ETYA, but not by indomethacin, led to our early speculation that EDRF may be a product of lipoxygenase oxidation of arachidonic acid (or some other unsaturated fatty acid) liberated from phospholipids of the endothelial cells (Furchgott and Zawadzki, 1980; Furchgott et al., 1981; see section VII). With this speculation in mind, Zawadzki, Cherry, and I carried out a number of experiments testing arachidonic acid (AA) on rings or rabbit aorta. Usually, but not always, this agent, at concentrations of 10–100 μM, produced moderate relaxation of NE-induced contractions that was dependent on endothelial cells and not only was not inhibited, but, actually, was potentiated, by indomethacin. Singer and Peach (1983a) have independently demonstrated the endothelium-dependent relaxation of rings of rabbit aorta by AA, and its potentiation by indomethacin. They found that preincubation of rings with ETYA (50 μM) or NDGA (25 μM) for 15 minutes completely prevented relaxation by AA. On the other hand, we find that neither ETYA nor NDGA added during relaxation of rabbit aorta by AA acutely antagonizes the relaxation, as it does in the case of relaxations by ACh or A23187. Recently, De Mey and co-workers have reported their findings on relaxation of rings of canine arteries by AA (De Mey et al., 1982; De Mey and Vanhoutte, 1982). Relaxation of splenic artery was completely lost after removal of the endothelium, and relaxations of pulmonary, saphenous, and femoral arteries were reduced markedly, especially at lower concentrations of AA (0.1–3 μM). Thus, relaxation of these dog arteries by AA is largely endothelium-dependent (De Mey and Vanhoutte, 1982). In the case of the femoral artery with endothelium, it was found that indomethacin (20 μM for 60 minutes) and ETYA (100 μM for a shorter time) both completely inhibited relaxation by AA at concentrations up to 30 μM (De Mey et al., 1982). These workers also used thin-layer chromatography to analyze for products formed by isolated femoral arteries incubated with [³⁵S]AA. A major product in endothelium-containing preparations was 6-keto-PGF₁α, the prostaglandin to which PGI₂ is spontaneously converted. This product was almost completely eliminated by either removing the endothelium or by pretreating the preparations with indomethacin or ETYA. Another product was a hydroxy derivative of AA that was probably formed by a lipoxygenase pathway and was much reduced in the absence of endothelium. De Mey and co-workers concluded from their results that relaxation of the dog femoral arteries by AA is
mediated by PGI₂, mainly produced by the endothelial cells from the added AA. In a very recent paper (De Mey and Vanhoutte, 1983), these investigators report that the relaxation of the canine femoral artery by AA is not inhibited by anoxia, but is actually potentiated to some extent. This is quite unexpected, for one would hardly expect PGI₂ to be formed from AA in the absence of oxygen.

In my laboratory, we have also investigated the endothelium-dependent relaxation of isolated canine coronary and superior mesenteric arteries by AA. The cyclooxygenase inhibitors, indomethacin and flurbiprofen, blocked relaxation produced by low concentrations (0.1–1 μM), but not by high concentrations (10–100 μM) of AA (Cherry et al., 1983). In these dog arteries, after cyclooxygenase inhibition, as in rabbit aorta, ETYA and NDGA failed to acutely antagonize AA-induced relaxation. Moreover, it has been found that other unsaturated fatty acids beside AA (e.g., cis-4,7,10,13,16,19-docosahexanoic, oleic, elaidic, and cis-vaccenic) can produce relaxations in these dog and rabbit arteries that are endothelium-dependent and not blocked by cyclooxygenase inhibitors (Cherry et al., 1983). Since some of these unsaturated fatty acids have been shown by others to increase the fluidity of cell membranes to which they have been added and to enhance the rate of certain enzymatic reactions in membranes (Orly and Schramm, 1975; Rimon et al., 1978), it has been proposed that endothelium-dependent relaxation by these unsaturated fatty acids may be the result of an increase in fluidity of the endothelial cell membrane that facilitates reactions leading to formation of a relaxing factor. The finding that relaxation by these agents (including AA in the presence of a cyclooxygenase inhibitor) is enhanced at lower temperatures (22° vs. 37°) is thought to be consistent with the concept that they act by changing membrane fluidity (Cherry et al., 1981).

V. Role of Endothelial Cells in the Relaxation of Veins and the Vasodilation of Resistance Vessels

A. Veins

In longitudinal strips of rabbit portal vein brought to moderate tone with NE, 100 nM ACh evoked a little relaxation, while 1 μM and higher produced contraction (Furchgott et al., 1981). Recently, De Mey and Vanhoutte (1982) reported their findings on isolated rings of various canine veins precontracted with NE: in femoral and saphenous veins, ACh in the range of 30–300 nM produced relatively small endothelium-dependent relaxations (maximum of 20–25% of the NE tone); in the pulmonary vein, ACh produced no relaxation but only contraction, whether or not endothelial cells were present; and in the splenic vein, ACh in the range of 10–100 nM produced moderate relaxations which appeared to be endothelium-independent. Thus, the veins, on the whole, gave much less relaxation in response to ACh than did the corresponding arteries. The endothelium-independent relaxation of the splenic vein is puzzling. The absence of, or limited, relaxation in the case of the other veins might be partly explained by a masking of endothelium-dependent relaxation by a strong direct contracting effect of ACh on the venous muscle at higher concentrations (De Mey and Vanhoutte, 1982). However, this is not a full explanation, for the canine femoral vein, which gives only small endothelium-dependent relaxations with ACh and with BKN, also gives only small endothelium-dependent relaxation with A23187 (unpublished observations). The reason for the much smaller endothelium-dependent relaxation of canine veins, as compared to arteries, by ACh, BKN, and A23187, is not clear at present.

De Mey and Vanhoutte (1982) also investigated the effects of ATP, bovine thrombin, and arachidonic acid (AA) on corresponding canine veins and arteries (pulmonary, femoral, splenic, and saphenous) precontracted by NE. Unlike the arteries, which exhibited good to excellent endothelium-dependent relaxation to all of these agents, the veins exhibited either no relaxation or transient relaxation of small degree. In the case of thrombin and AA, it appears unlikely that an endothelium-dependent relaxation was being masked by a contracting action of these agents on the veins, for the increased levels of contraction (that is, above the level produced by the pre-added NE) produced by these two agents was not greater but actually smaller in veins that had been denuded of their endothelial cells.

B. Resistance Vessels

In our first paper on the endothelium-dependent mechanism for relaxation of isolated arteries by ACh, we proposed that this mechanism is also applicable in the case of vasodilation of smaller resistance vessels (arterioles), and that it is one of two principal mechanisms responsible for the very potent vasodilating effect of ACh on these vessels in intact animals (Furchgott and Zawadzki, 1980b). The other mechanisms to which we referred is that in which ACh, acting on prejunctional muscarinic receptors of adrenergic nerves, inhibits the stimulation-evoked release of NE from these nerves, and thus the vasoconstricting resulting from nerve stimulation. (For review, see Vanhoutte, 1977; Fozard, 1979). It was emphasized that the endothelium-dependent mechanism for vasodilation by ACh, unlike the prejunctional inhibitory mechanism, would be effective on resistance vessels whether or not the vasoconstriction was the result of adrenergic nerve stimulation.

In preliminary studies, we have obtained evidence that is consistent with the proposal that ACh dilates resistance vessels by acting on endothelial cells (Carvalho and Furchgott, 1981). Our preparation was the perfused isolated arterial vasculature of the rabbit mesentery, a modification (Blumberg et al., 1977) of the preparation originally introduced by McGregor (1965) for rat mesenteric vasculature. Oxy-
genated Krebs-bicarbonate solution at 37°C was perfused into the superior mesenteric artery at a constant rate of flow. Vasodilation of the perfused preparation, preconstricted by continuous perfusion of NE, was produced by additions of ACh (0.1-1 μM) or A23187 (0.01-0.1 μM) to the perfusion fluid, and was still pronounced even when a cyclooxygenase inhibitor (indomethacin, 28 μM, or flurbiprofen, 33 μM) was present. In two experiments, the vasodilator responses were measured before and after perfusion with collagenase at the concentration (0.2%) and for the period of time (15 minutes) that had successfully removed endothelial cells and eliminated ACh-induced relaxation in rabbit aortic rings (Furchgott and Zawadzki, 1980b). The collagenase perfusion resulted in elimination of the vasodilator response to ACh; and reduced, but never eliminated completely, the vasodilator response to A23187 (Carvalho and Furchgott, 1981). The duration of experiments of this type was limited because basal resistance to flow tended to increase after collagenase treatment, probably because of edema formation.

Another finding that supports the proposal that vasodilatation of resistance vessels by ACh is endothelium-dependent comes from recent work of M. Owen (personal communication). She found that branches of rabbit ear artery as small as 150 μm o.d., mounted as rings in vitro, show endothelium-dependent relaxation by ACh, and are even more sensitive than the central ear artery.

VI. Possible Facilitation of Contraction of Blood Vessels by Endothelial Cells

De Mey and Vanhoutte (1982), in a recent study on the behavior of isolated canine arteries and veins (femoral, splenic, saphenous, pulmonary), concluded that endothelial cells under certain conditions can facilitate the contraction of vascular smooth muscle. This conclusion was based on the findings that mechanical removal of the endothelium: (1) reduced the maximal contractile response to NE in most of the blood vessels tested; (2) reduced in veins the increase in tension produced by thrombin, and reduced or abolished the increase produced by arachidonic acid; (3) reduced or reversed the increase in tension caused by anoxia introduced during NE-induced contraction. The authors do not speculate on how removal of endothelial cells reduced the maximal response to NE and reduced the increases in tension in veins evoked by thrombin or arachidonic acid. It would be interesting to determine whether these increases in tension in the presence of endothelium are influenced by inhibition of cyclooxygenase. The endothelium-facilitated increase in tension (potentiation) evoked by the introduction of anoxia on vessels during stimulant-induced tone has been studied further by De Mey and Vanhoutte (1983) in the case of the canine femoral artery. They find that inhibition of cyclooxygenase by indomethacin does not prevent the potentiating response of anoxia on NE-induced tone. They proposed that the facilitation by endothelium of this anoxic potentiation is likely caused by either the generation of a facilitating modulator or the interruption of the production of an endogenous inhibitory substance by anoxia.

In connection with the possibility of a role for endothelial cells in facilitating contraction, it may be recalled that Bevan and Duckles (1975) found that the placement of "norepinephrine beads" [glass beads, 20–300 μm in diameter, with NE covalently bound (Venter and Kaplan, 1974)] on the upward-facing intimal surface of helical strips of rabbit aorta produced contraction of the strips. With strips that had been rubbed on their intimal surface with an aluminum sulfate "styptic" stick (in order to damage the intimal cells), the contraction obtained with beads was almost completely eliminated, whereas that obtained with NE in solution was still about 50% of the pretreatment level. The results of their experiments led Bevan and Duckles to propose that either NE attached to the beads, or NE released locally in high concentrations close to the bead surfaces, acted on α-adrenergic receptors on the intimal surface of the endothelial cells, creating a stimulus that was spread by some unknown mechanism from the endothelial cells to the subjacent smooth muscle, causing the latter to contract. This proposal is interesting, but recent findings raise some doubts about it. First, we know that when endothelial cells are carefully removed (mechanically or enzymatically) from strips (or rings) of rabbit aorta, there is no loss of sensitivity of the preparations to the contracting action of added NE (see Fig. 1), as would be expected if endothelial cells mediated a stimulus for contraction. Second, it seems likely that most of the endothelial cells would have been rubbed off inadvertently during the preparation of the helical strips (Furchgott and Zawadzki, 1980b; Furchgott, 1982). Third, it has been demonstrated in rabbit aorta that the muscle cells of the media nearer the intima are considerably more sensitive to the contracting action of NE than are those nearer the adventitia (Pascaul and Bevan, 1980). In view of these recent findings, one must now consider the possibility that—in the experiments of Bevan and Duckles—the cells on which NE from the beads acted were not the endothelial cells but, rather, the sensitive smooth muscle cells nearest the intimal surface. Whether the observed contractions were limited to these cells, or whether there was also a spread of a stimulus for contraction by some unidentified mechanism from the outermost smooth muscle cells to cells deeper in the tunica media, cannot be decided at present.

VII. The Nature of EDRF Released by Acetylcholine and Other Agents

EDRF has not yet been identified chemically. Our early work ruled out any prostaglandin, adenosine, or AMP as the active factor (Furchgott and Za-
wadzki, 1980b). The inhibition of ACh-induced relaxation of rabbit aorta by anoxia, ETYA (an inhibitor of lipoxygenase), and quinacrine (an inhibitor of release of arachidonic acid from certain phosphatides) (see Section II, A–C) suggested that ACh, acting on the muscarinic receptor of the endothelial cells, somehow activates a reaction sequence in which arachidonic (or some other unsaturated fatty acid) is liberated from phosphatides and then oxidized by lipoxygenase, but by inhibiting a step or steps in this latter finding with the early speculation was several later findings, namely, the inhibition of ACh-induced relaxation by hydroquinone (a potential free radical scavenger), by NDGA (a lipoxygenase inhibitor and antioxidant), and by BPB (an inhibitor of phospholipase A2) (see Section II, D–F). However, all of these findings are not sufficient evidence for a firm conclusion that EDRF is an oxidation product formed via a lipoxygenase pathway, since each of the agents noted above could be acting to inhibit ACh-induced relaxation by some mechanism other than the one that is parenthetically cited.

One finding that appears to be inconsistent with our speculation about the nature of EDRF is that the lipoxygenase inhibitor BW755C does not inhibit ACh-induced relaxation in rabbit aorta (Furchgott and Wadzki, 1980b). However, the possibility exists that BW755C, although capable of inhibiting lipoxygenase activity in some cells (Higgs et al., 1978), is not an effective inhibitor of a specific endothelial lipoxygenase involved in EDRF formation. The findings on the endothelium-dependent relaxation of rabbit aorta by arachidonic acid (AA) during cyclooxygenase inhibition (Section IV–F) are both consistent and inconsistent with the speculation—for example, the finding by Singer and Peach (1983a) that preincubation with NDGA or ETYA prevents AA-induced relaxation is consistent—although it should be noted that they postulate that these two agents may be inhibiting the oxidation of AA to an active product by cytochrome P450 rather than by a lipoxygenase. On the other hand, our finding that ETYA or NDGA added during the course of an AA-induced relaxation does not acutely antagonize the relaxation (as in the case of relaxation by ACh) is inconsistent with the speculation concerning the nature of EDRF. One way to reconcile this latter finding with the early speculation would be to assume that both ETYA and NDGA acutely antagonize ACh-induced relaxation, not by inhibiting lipoxygenase, but by inhibiting a step or steps between the muscarinic receptor stimulation and the liberation of AA (or another unsaturated fatty acid) from endogenous phosphatides. However, this assumption is contrary to the assumptions which led to the proposal in the first place. The possibility therefore exists that AA releases a nonprostaglandin EDRF different from that released by ACh. It is also conceivable that both ACh, acting on its receptor, and exogenous AA, interacting with the membrane of the endothelial cell, alter the cells so as to allow the activation of some common, ordinarily restrained reaction sequence which gives rise to an EDRF that is not itself an oxidation product of an unsaturated fatty acid. The recent finding that some unsaturated fatty acids other than AA also produce endothelium-dependent relaxation of dog and rabbit arteries (Cherry et al., 1983) has led to the proposal that a change in fluidity of the membrane of the endothelial cells may be a primary step in the activation of the reaction sequence leading to EDRF (see Section IV–F).

The EDRF released by A23187 and substance P in rabbit, canine, and feline arteries and by BKN in canine and human arteries is most likely the same substance (or substances) as the EDRF released by ACh in these arteries. This conclusion is based on the common susceptibilities to inhibitor agents of the relaxation produced by each of these agents and that produced by ACh in any given artery (Furchgott et al., 1983) (see Sections III and IV, B and C). On the same basis, we concluded that EDRF released by ATP and ADP in rabbit aorta is similar to that released by ACh. However, De Mey et al. (1982) concluded, that in the canine femoral artery, relaxation by ATP is mediated by a different "signal" (EDRF?) than is relaxation by ACh, since relaxation by ATP, unlike that by ACh, was not susceptible to inhibition of ETYA and quinacrine. De Mey and co-workers also found no inhibition of ETYA or quinacrine of the endothelium-dependent relaxation of the dog femoral artery by thrombin, and therefore concluded that this agent, too, produces a different "signal" for relaxation than does ACh.

The goal of chemically identifying EDRF that is released by ACh from endothelial cells will probably be difficult to achieve. To my knowledge, no one has yet been able to incubate endothelial cells (either attached to an arterial wall or in culture) with ACh (or A23187) and then demonstrate the presence of EDRF in the incubation mixture by bioassay on endothelium-free preparations of arteries. It is likely that EDRF is a very labile substance, and that special procedures will be required for producing, stabilizing, and purifying it.

VIII. Speculation on the Mechanism by Which EDRF Activates Relaxation

Prior to our findings that suggested that EDRF released by ACh may be a product of the oxidation of arachidonic or some other unsaturated fatty acid, other workers had reported that in certain smooth muscles there was a positive relationship between an increase in cGMP and relaxation (Katsuki and Murad, 1977; Böhm et al., 1978; Schultz et al., 1979; Murad et al., 1979), and that guanylate cyclase was markedly stimulated by hydroperoxides of arachidonic acid (Hidaka and Asano, 1977; Goldberg...
et al., 1978) and by free radicals, particularly nitric oxide and the hydroxyl radical. (For review, see Murad et al., 1979.) Murad and his colleagues had proposed that many potent vasodilators, such as nitroprusside, organic nitrates, azide, and inorganic nitrite, activate guanylate cyclase indirectly via nitric oxide which they release as a reaction product. These findings of others prompted us to speculate that (1) EDRF is either a short-lived hydroperoxide or free radical arising as an intermediate product in the oxidation of liberated AA by the lipoxygenase pathway, and (2) EDRF, after diffusing from the endothelial cells to the smooth muscle cells of the artery, stimulates the muscle guanylate cyclase, causing an increase in cGMP which then somehow activates relaxation (Furchgott et al., 1981). This speculation assumes a causal relationship between increases in cGMP levels and relaxation in vascular smooth muscle. Evidence consistent with such a causal relationship in the case of relaxation of isolated bovine coronary arteries by nitric oxide and nitric oxide-releasing drugs has recently been presented in several reports (e.g., Kukovetz et al., 1979; Ignarro et al., 1981; Gruetter et al., 1981). However, admittedly, the case for a causal relationship has not yet been proven, and there is as yet no generally accepted hypothesis about a mechanism by which cGMP might produce relaxation.

Rapport and Murad (1983) recently obtained results in accord with our speculation that EDRF stimulates an increase in vascular smooth muscle cGMP. They used spiral strips of rat thoracic aorta, incubated in Krebs bicarbonate solution. Some strips were exposed to the contracting agent NE (0.1 \(\mu\)M) alone, while others were exposed to NE plus either ACh (10 \(\mu\)M), histamine (100 \(\mu\)M), or A23187 (3 \(\mu\)M) for varying times prior to freezing in liquid N\(_2\). These concentrations of ACh, histamine, and A23187 had been found optimal for endothelium-dependent relaxation of NE contractions. With NE alone, cGMP usually averaged about 0.8 pmol/mg protein in intact strips (endothelium present) and about 0.5 pmol/mg protein in endothelium-free strips. ACh, histamine, and A23187 all produced marked increases (20- to 40-fold) in cGMP in intact strips with peak levels being reached in 30 seconds, but produced no increases in endothelium-free strips. In contrast to these relaxants, nitroprusside (1 \(\mu\)M), which does not depend on endothelium for its relaxing effect, produced a mean peak increase in cGMP of more than 60 pmol/mg protein in both endothelium-free and intact strips. Rapoport and Murad (1983) believe that the increase in cGMP in intact strips after ACh is in the muscle rather than the endothelium, since a 5-second rubbing to remove endothelium at the end of a 30-second exposure of intact strips to ACh still left elevated levels of cGMP. ACh produced no significant changes in cAMP.

Recently, we have also obtained evidence that EDRF released by ACh and A23187 in rabbit aorta is associated with an increase in cGMP (Furchgott and Jothianandan, 1983). Rings of rabbit aorta were mounted to allow both recording of tension and rapid freezing in liquid N\(_2\) at any desired stage of contraction and relaxation. Basal values of cGMP (no drugs) averaged 0.20 pmol/mg protein in intact rings (endothelium present) and 0.06 in endothelium-free rings. During contractions by NE (usually 0.1 \(\mu\)M) there were no significant changes in these levels. Relaxation of NE contractions by ACh (1 \(\mu\)M) in intact rings was accompanied by a mean 5-fold increase in cGMP, whereas relaxation by A23187 (0.1 \(\mu\)M) was accompanied by a mean 7-fold increase. These increases, though less than those found by Rapoport and Murad in rat aorta, were still highly significant \((P < 0.001)\). On endothelium-free rings, ACh and A23187 produced neither relaxation of NE concentrations nor any change in cGMP. Nitroglycerin (0.44 \(\mu\)M), which relaxed endothelium-free rings as well as intact rings, produced marked increases in cGMP in both types of rings. None of the drugs tested produced any significant changes in cAMP in either intact or endothelium-free rings (mean control levels about 1.0 pmol/mg protein in both).

A role for hyperpolarization of membranes during ACh-induced relaxation has been considered. Venet et al. (1975) found that ACh produced hyperpolarization of the membranes of cultured endothelial cells in an established line from rabbit aorta. Also, Kuriyama and Suzuki (1978), in a study of membrane and contractile properties of segments and strips of rabbit superior mesenteric artery, found that ACh at low concentrations hyperpolarized the muscle cell membranes; but they concluded from their results that ACh-induced relaxation during either K\(^+\)-induced or NE-induced contraction (found in adult but not in young rabbits) was not caused by hyperpolarization of the membranes. Their study was carried out before the discovery of the obligatory role of endothelial cells in the relaxation of arteries by ACh. The possibility that the endothelium-mediated relaxation of rabbit aorta by ACh requires hyperpolarization of the smooth muscle cells was eliminated when we found that intact aortic rings which had contracted in a completely depolarizing \(K_2\)SO\(_4\)-Krebs solution still relaxed in response to ACh (Furchgott and Zawadzki, 1980b; also see remarks in Section I-B). Admittedly, this finding does not rule out the possibility that hyperpolarization may make some contribution to the endothelium-mediated relaxation in physiological solutions, but there is at present no evidence for this. On the other hand, there is strong evidence against the proposal of Chand and Altura (1981b) that the relaxation of dog pulmonary arteries of ACh or bradykinin is due to hyperpolarization of the endothelial cell membrane, followed by a transfer of hyperpolarizing current through myoendothelial junctions to the underlying vascular smooth muscle.
For example, in our 'sandwich experiments' (see Section I-C and Fig. 2), in which relaxation occurs in the endothelium-free recipient strip of artery, there can be no transfer of current because there are no myoendothelial junctions between the muscle cells of that strip and the endothelial cells of the donor strip.

IX. Concluding Remarks

The agents which by now have been shown to produce relaxation of certain isolated mammalian arteries by stimulation of release of a nonprostaglandin relaxing factor (or signal) from the endothelial cells include some of the most potent endogenous vasodilators found in mammals—namely, ACh, bradykinin, ATP, and ADP, substance P, and histamine (acting on an H₁-receptor). Not included are adenosine, prostaglandins such as PGI₂ and PGE₂, histamine (acting on an H₂-receptor), and epinephrine and norepinephrine (acting on β-adrenergic receptors), all of which are endogenous vasodilators that appear to act directly on the vascular smooth muscle. In ascribing the relaxing effect of any agent to the stimulation of release of a nonprostaglandin endothelium-derived relaxing factor (EDRF), it is important to specify the species and the arteries in which this mechanism was experimentally demonstrated. This precaution is important, since the same agent may produce relaxation by different mechanisms in different species (as in the case of bradykinin) or in different arteries of the same species (as in the case of histamine). Indeed, there is the possibility that more than one mechanism may contribute to relaxation in a single artery (e.g., see Section IV-A, on the endothelium-dependent and endothelium-independent mechanism for relaxation by ATP). Although we have attributed the relaxation of the rabbit superior mesenteric artery by bradykinin to an endothelium-independent release of prostaglandin from the vessel wall (Section IV-B), we have recently encountered one preparation of this artery that exhibited relaxation by bradykinin that appeared to be mediated by both the prostaglandin mechanism and the endothelium-dependent nonprostaglandin mechanism. In the case of ACh and substance P, in all arteries of all species so far tested, the relaxation of drug-induced tone has appeared to be solely the result of their stimulation of release of EDRF.

It is still too early to evaluate the physiological significance of relaxation of blood vessels that is endothelium-dependent and not mediated by prostaglandins. Before such an evaluation can be made, it must be ascertained whether this indirect mechanism for relaxing vascular smooth muscle is involved in the actions of endogenous vasodilators such as ACh, ATP, substance P, bradykinin, and histamine on resistance vessels (arterioles) that modulate blood flow in various vascular beds. If this mechanism is involved, then loss of the capacity of endothelial cells in a given vascular bed to generate EDRF in response to one or more of these endogenous vasodilators may well lead to compromise of blood flow in that bed. Already, there has been speculation based on this concept. For example, Chand and Altura (1981b) speculated that damage to the endothelial cells in the pulmonary vascular bed might allow endogenous ACh and BKN to now increase rather than decrease resistance to flow, and thus contribute to the development of both pulmonary hypertension and shock lung. Also, Ku (1982), after finding that the endothelium-dependent relaxation but not the contraction elicited by thrombin in isolated canine coronary arteries was largely lost in arteries that had been occluded in situ for 90 minutes before reperfusion and removal, speculated that altered responses to thrombin of coronary arteries with endothelium damaged by acute ischemia may play an important role in the pathogenesis of coronary vasospasm.

In addition, consideration should be given to the possibility that certain drugs—already developed or yet to be developed—may produce vasodilation by acting via endothelial cells. Indeed, Spokes et al. (1983) have very recently reported that, in rabbit aortic rings, the relaxant effect of the antihypertensive drug hydralazine is dependent in part on the presence of endothelium, whereas the effects of minoxidil, diazoxide and nitroprusside are not.

Regardless of future developments on the physiological, pathological, and pharmacological significance of the release from endothelium of a nonprostaglandin relaxing factor, EDRF, or—perhaps—factors, since there is some evidence for a different factor or signal in the case of thrombin and possibly ATP, as compared to ACh, the chemical identification of the factor or factors remains a major research goal. As pointed out in Section VII, newer findings have cast some doubt on the original hypothesis that the EDRF released by ACh is an intermediary product of lipoxygenase oxidation of AA or some other unsaturated fatty acid. And, finally, directly related to the goal of identifying EDRF is the other major goal of determining the mechanism by which it activates relaxation of vascular smooth muscle.

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