J. Physiol. (1981), **316**, *pp.* 347–355
With 5 text-figures
Printed in Great Britain

ROLE OF THE INTIMA IN CHOLINERGIC AND PURINERGIC RELAXATION OF ISOLATED CANINE FEMORAL ARTERIES

BY J. G. DE MEY AND P. M. VANHOUTTE

From the Division of Pharmacology, Faculty of Medicine, Universitaire Instelling Antwerpen, Wilrijk, Belgium

(*Received 10 October 1980*)

SUMMARY

1. Experiments were designed to determine the role of the endothelium in relaxations of isolated blood vessels caused by ACh, adenine nucleotides and K^+ ions.

2. Paired rings of canine femoral arteries were mounted for isometric tension recording in organ chambers filled with aerated Krebs–Ringer solution (37 °C). One ring served as control; in the other ring the intimal layer was removed mechanically.

3. Removal of the endothelium only slightly depressed the maximal contractile response to noradrenaline, and did not affect the apparent sensitivity to the catecholamine. It depressed the contractile response to 25–60 mM-K^+ more than that to noradrenaline.

4. In the absence of endothelium, the femoral arteries did not relax on exposure to ACh.

5. Removal of the endothelium did not affect relaxations caused by adenosine and AMP, but markedly reduced those caused by ADP and ATP.

6. The relaxations induced by 5·9 mM-K^+ were comparable in control rings and arteries denuded of their endothelium.

7. These experiments demonstrate that in the canine femoral artery, relaxations induced by ACh, ADP and ATP require the presence of functional endothelial cells, which, when exposed to these substances, initiate an inhibitory signal for the smooth muscle cells of the media. By contrast, relaxations of isolated arteries caused by adenosine, AMP and K^+ ions must be due mainly to a direct effect on the vascular smooth muscle cells.

INTRODUCTION

Besides its role in capillary transport, the endothelium releases prostacyclin which inhibits platelet aggregation, and removes certain vasoactive substances from the blood (see Bakhle & Vane, 1974; Moncada & Vane, 1978; Shepherd & Vanhoutte, 1979; Gillis, 1980). In addition, there are indications that the intimal side of the blood vessel wall can recognize signals which affect the function of the smooth muscle cells of the media. Thus, it has been suggested that contact of noradrenaline with endothelial cells can cause the smooth muscle to contract before the amine has permeated into the vascular wall (Bevan & Duckles 1975; Pascual & Bevan, 1980) and that the endothelium plays an obligatory role in the relaxation of isolated arteries

caused by acetylcholine (Furchgott & Zawadzki 1980). The present experiments were designed to determine the involvement of intimal structures in the relaxation of the isolated canine femoral artery evoked by ACh, adenosine nucleotides and potassium ions. Earlier work has shown that this preparation is very sensitive to the relaxing effects of these substances (Vanhoutte, 1974; De Mey & Vanhoutte, 1980*a*).

METHODS

The experiments were performed on paired rings (5 mm width) of femoral arteries taken from dogs (20–38 kg) anaesthetized with pentobarbitone (30 mg/kg; I.V.). One of the rings served as control; the other ring was inverted to expose the endothelium which was removed by mechanical rubbing (Furchgott & Zawadzki 1980), and the ring was returned to its original configuration prior to experimentation.

Isometric tension recording

The rings were attached to isometric force transducers (Statham UC3) and suspended in individual organ chambers filled with Krebs–Ringer bicarbonate solution (composition mM: NaCl, 118·3; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4 . 7H_2O$, 1·2; KH_2PO_4, 1·2; $NaHCO_3$, 25·0; Ca EDTA, 0·026; glucose, 11·1). The solution was maintained at 37°C and gassed with 95% O_2–5% CO_2 (pH 7·4). Prior to experimentation, the segments were stretched to the optimal point of their length–tension relationship using a standard concentration of noradrenaline (5×10^{-7} M); the optimal tension averaged $18·1 \pm 0·9$ g for the control preparations and $17·3 \pm 1·1$ g for the rings from which the endothelium was removed ($n = 18$). After this procedure, the rings were allowed to equilibrate for 45 min. Changes in the K^+ concentration (5·9–120 mM) of the Krebs–Ringer solution were compensated for by equimolar adjustment of the Na^+ concentration. To obtain potassium-free solutions, KCl and KH_2PO_4 were replaced by equimolar amounts of NaCl and NaH_2PO_4, respectively.

Drugs

The drugs used were acetylcholine hydrochloride (ACh; Sigma), adenosine (Sigma); adenosine monophosphate (AMP; Sigma), adenosinediphosphate (ADP; Sigma), adenosine triphosphate (ATP; Sigma), 1-noradrenaline bitartrate (Fluka), phentolamine mesylate (Ciba), and theophylline (Boehringer). All concentrations are expressed as final bath concentrations (M).

Statistical analysis

Each experimental group consisted of six preparations taken from six dogs. For statistical analysis Student's *t* test for paired and unpaired observation was used. When P was smaller than 0·05 values were considered to be significantly different. Only significant changes are discussed in the Results section.

Histology

To ascertain that the mechanical rubbing applied to the femoral rings had successfully removed the endothelium, control and rubbed rings were incubated for 2 hr in Krebs–Ringer solution at 37°C. They were opened longitudinally and stained *in vitro* with $AgN0_3$ as described by Caplan, Gerrity & Schwartz (1974). Briefly, the preparations were immersed successively in the dark at room temperature in: (1) Hepes (20 mM) buffered (pH 7·4) solution containing 4·6% glucose for 150 sec; (2) 0·4% $AgN0_3$ in 4·2% glucose solution for 60 sec and (3) 4·6% glucose solution for 60 sec. The arteries then were fixed at room temperature in 0·1 M-sodium cacodylate containing 7·5% sucrose. Light microscopic examination of the luminal surface of the control rings revealed a mosaic pattern of silver lines (Fig. 1*A*), which are considered to represent the borders of adjacent endothelial cells (Caplan *et al.* 1974); this mosaic pattern was not seen in arteries where the intimal layers had been rubbed mechanically (endothelium-denuded rings; Fig. 1*B*), indicating that the procedure had removed the endothelial layer.

RESULTS

Contractile responses

Both control and endothelium-denuded rings contracted in a dose-dependent manner when exposed to increasing concentrations of noradrenaline. To judge from the dose–response curves, the sensitivity of the preparations to the catecholamine was comparable (Fig. 2). However, the maximal absolute increase in tension caused

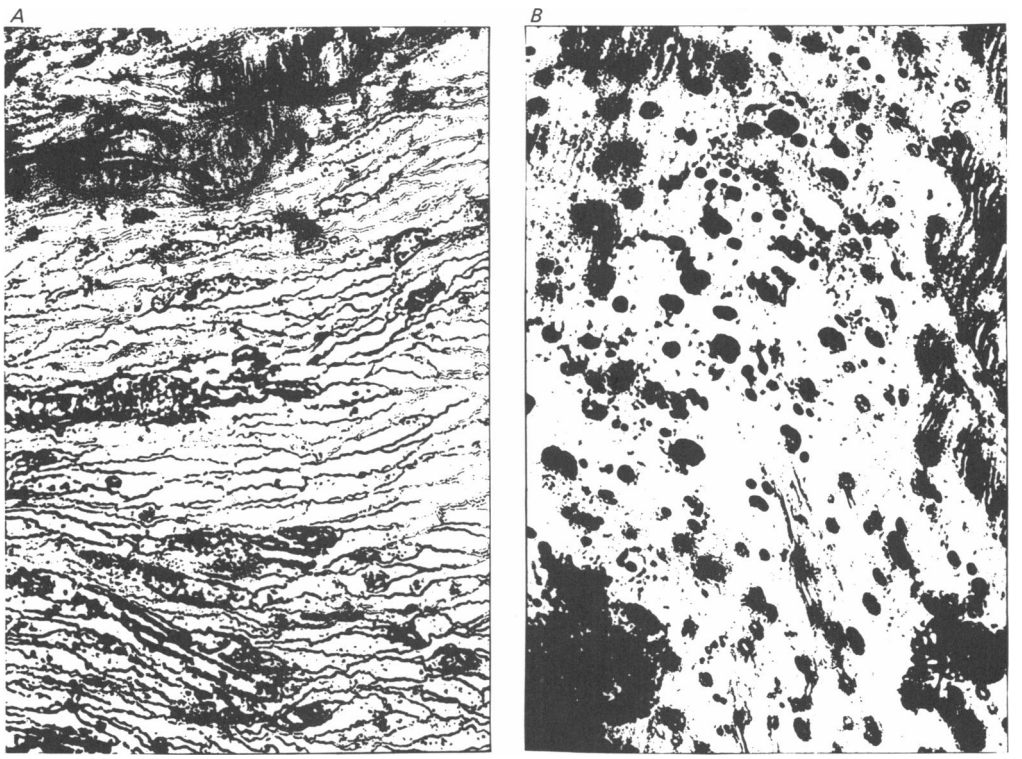

Fig. 1. Silver nitrate staining of intimal side of canine femoral artery without (*A*) and after (*B*) rubbing of the intimal side. (Courtesy of Dr M. Borgers.)

by noradrenaline, expressed per cross-sectional area, was greater in control rings than in endothelium-denuded arteries ($24{\cdot}4 \pm 1{\cdot}4$ g/mm^2 and $18{\cdot}9 \pm 3{\cdot}9$ g/mm^2, respectively).

In presence of 5×10^{-6} M-phentolamine, increasing amounts of K^+ caused concentration-dependent increases in tension which, when expressed as percent of the response to noradrenaline, in the range of 25–60 mM-K^+, were significantly smaller in the endothelium-denuded rings than in the control preparations (Fig. 2).

Acetylcholine

When control rings were made to contract with 10^{-7} M-noradrenaline or 25 mM-K^+ in presence of 5×10^{-6} M-phentolamine), the addition of ACh caused dose-dependent

(10^{-9}–10^{-6} M) relaxations; further increases in the ACh concentration did not cause additional changes in tension; the amplitude of the relaxation caused by ACh was comparable in control solution and in Krebs–Ringer solution containing 6×10^{-6} M-physostigmine. In endothelium-denuded rings, made to contract (in absence and presence of 6×10^{-6} M-physostigmine) with either 10^{-7} M-noradrenaline or 25 mM-K^+ (in presence of phentolamine). ACh did not evoke relaxations (Fig. 3).

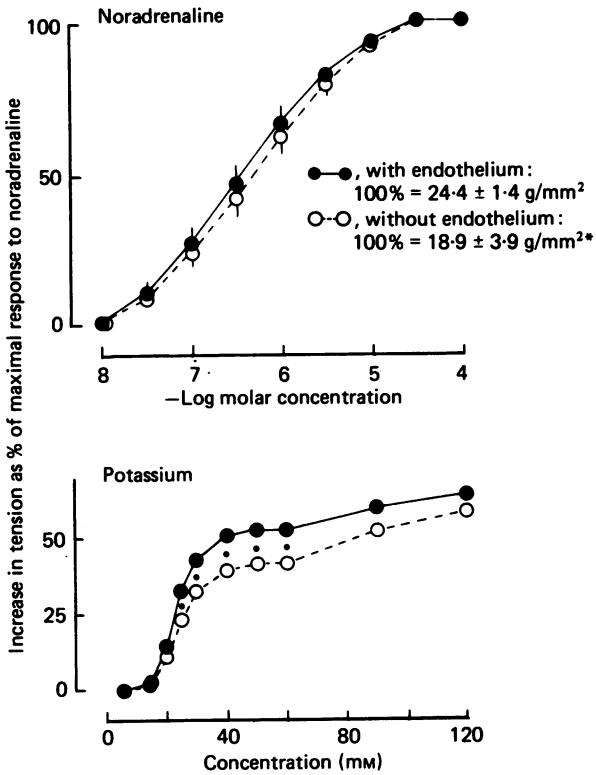

Fig. 2. Comparison in six isolated canine femoral arteries of the response to noradrenaline (upper) and K^+ (lower). Control preparations (●) were compared with paired rings of the same arteries where the endothelium had been mechanically removed (○). The increases in tension are expressed as percent of the individual maximal response to noradrenaline and shown as means ±S.E. of means *: difference from control rings is statistically significant ($P < 0.05$).

Adenosine and adenine nucleotides

Control rings, made to contract with 10^{-7} M-noradrenaline, relaxed when exposed to increasing concentrations of adenosine, AMP, ADP and ATP; the order of potency of the adenine nucleotides was: ATP = ADP > AMP = adenosine; at 10^{-4} M, ATP depressed the contractile response to noradrenaline by 95·6±7·3%. Removal of the endothelium did not significantly affect the responsiveness of the femoral artery to adenosine and AMP, but significantly reduced that to ADP and ATP (Fig. 4).

When control rings were made to contract with 25 mM-K^+ (in presence of 5×10^{-6}

M-phentolamine) the addition of ATP, but not of adenosine, caused dose-dependent (10^{-6}–10^{-4} M) relaxations; the relaxation with 10^{-4} M-ATP averaged 48·6 ± 8·7 % during K^+-induced contractions. Removal of the endothelium abolished the inhibitory effect of ATP.

In control rings made to contract with 10^{-7} M-noradrenaline the presence of 3×10^{-5} M-theophylline abolished relaxations induced by 5×10^{-4} M-adenosine, but did not affect those caused by 10^{-5} M-ATP; the latter relaxations averaged 49·3 ± 10·0 and 47·4 ± 10·1 % in the absence and presence of theophylline, respectively.

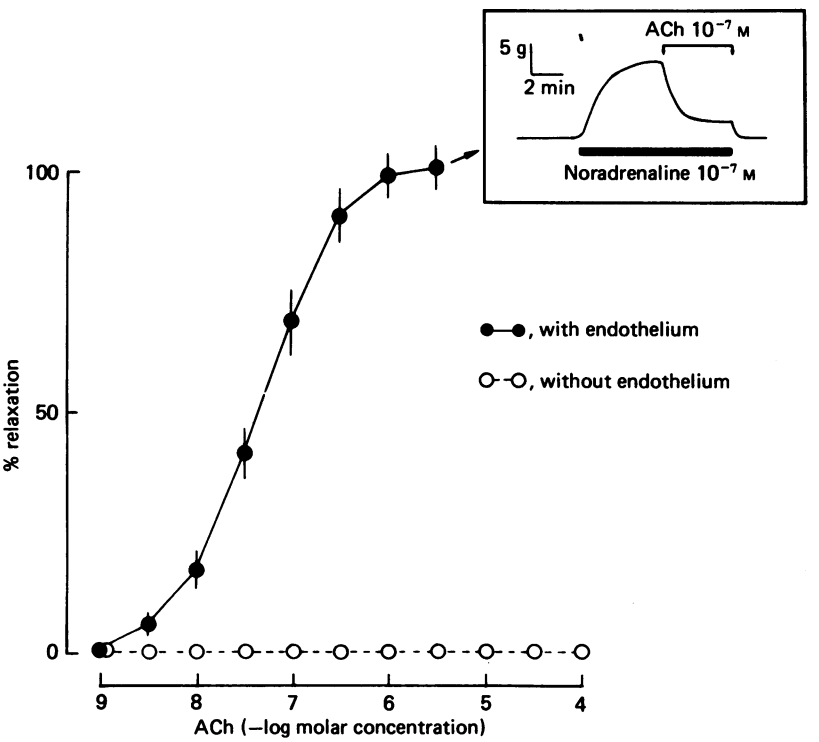

Fig. 3. Inset: isometric tension recording in an isolated canine femoral artery with endothelium intact. Effect of ACh during response to exogenous noradrenaline. Graph: comparison of the relaxing properties of increasing concentrations of ACh in paired rings of the same arteries ($n = 6$) with (●) and without (O) endothelium. The relaxations are expressed as percent depression of the control response to 10^{-7} M-noradrenaline (mean increase in tension: 13·2 ± 0·7 and 10·1 ± 0·8 g for preparations with and without endothelium, respectively) and shown as means ± S.E. of means.

Potassium

In both control and endothelium-denuded preparations, when incubated for 40 min in K^+-free solution and made to contract with noradrenaline (10^{-7} M), the addition of 5·9 mM-K^+ caused transient relaxations (Fig. 5). The relaxation induced by K^+ was complete and its time course comparable in both types of preparations (Fig. 5).

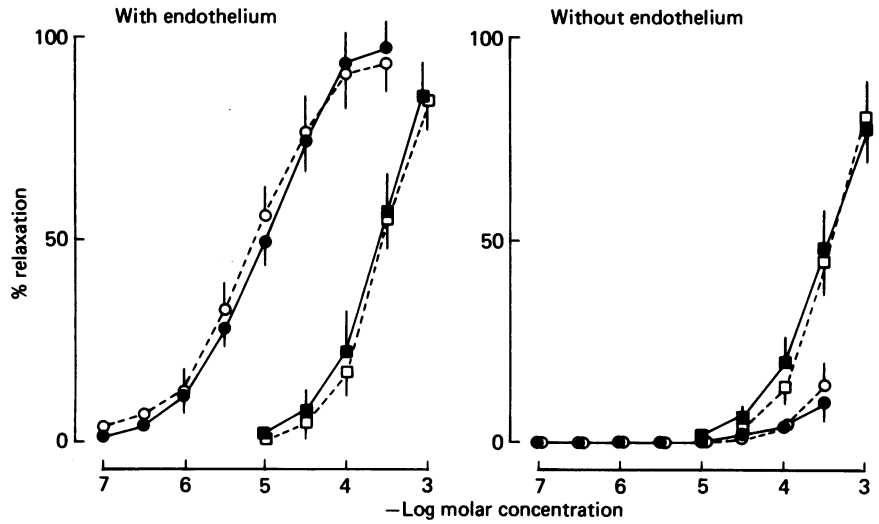

Fig. 4. Comparison in paired rings of canine femoral arteries ($n = 6$) with endothelium intact (left) or mechanically removed (right) of the relaxation induced by adenosine (□), AMP (■), ATP (●) and ADP (○) during contractions evoked by 10^{-7}M-noradrenaline (mean increase in tension: 13·7±0·8 (left) and 10·9±1·1 g (right). Relaxations are expressed as percent and shown as means±S.E. of means. All values obtained with ATP and ADP in rings without endothelium are significantly different from those noted in control preparations ($P < 0.05$).

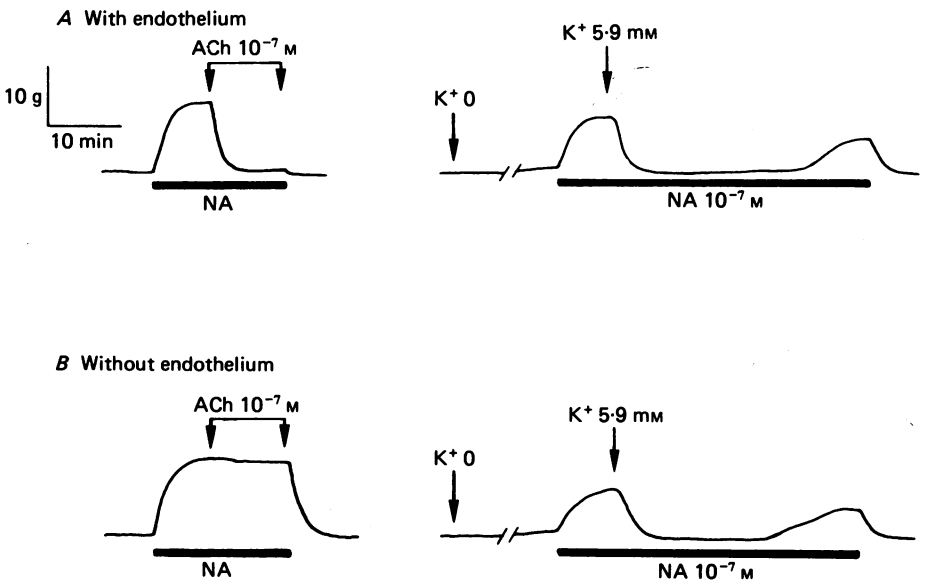

Fig. 5. Isometric tension recording of a pair of rings of the same femoral artery showing that a preparation with the endothelium intact (*A*) relaxes to both ACh and K^+ when made to contract with noradrenaline (NA), while a preparation from which the endothelium was mechanically removed (*B*) fails to respond to ACh, but shows a transient relaxation to K^+ comparable to that observed in the control ring.

DISCUSSION

The reduction of the contractile response to noradrenaline without alteration in the apparent sensitivity to the amine, observed after removal of the endothelium, is most likely due to slight mechanical damage of the smooth muscle cells during the preparation. The small size of the reduction rules out any major role of the endothelium in mediating the contractile responses to noradrenaline (Bevan & Duckles, 1975; Pascual & Bevan, 1980). Whereas the inhibitory effect of moderate increases in K^+ (Haddy, 1978; Webb & Bohr, 1978; De Mey & Vanhoutte, 1980*a*) is not affected by removal of the endothelium, the contractions caused by higher concentrations of the ion were depressed more by the procedure than those evoked by noradrenaline, suggesting that in normal rings the release of an endogenous vasoconstrictor from the intima contributes to the contractile response to high K^+.

Furchgott & Zawadzki (1980) have shown in isolated blood vessels of different species that removal of the endothelium abolishes the relaxation induced by the cholinergic transmitter. The present study confirms those findings for the femoral artery of the dog. The slightness of the innervation of this vessel (De Mey & Vanhoutte, 1980*a*) and the fact that physostigmine did not restore the response, make it very unlikely that either nerves or increased rate of breakdown of ACh played any part in the loss of the inhibitory effect of ACh on removing the endothelium. These experiments therefore strongly suggest that intimal structures, presumably endothelial cells, when exposed to ACh, can generate an inhibitory signal which is passed on to the vascular smooth muscle cells of the media and which, to judge from earlier work on the femoral artery, indirectly involves $Na^+ - K^+$ exchanges (De Mey & Vanhoutte 1980*a*, *b*).

In cultured endothelial cells of the rabbit aorta, ACh has been shown to cause hyperpolarization and to augment the levels of cyclic nucleotides, indicating that the cholinergic transmitter can indeed affect the cellular function of the intimal structures of the blood vessel wall (Buonassisi & Venter, 1976; Venter, Buonassisi, Bevan, Heinemann & Bevan, 1975). Whether the signal from the endothelial cells reaches the smooth muscle cells through cell-to-cell conduction (e.g. Rhodin, 1967; Henderson, 1975; Pascual & Bevan, 1980) or by the release of chemical messenger(s) (Furchgott & Zawadzki, 1980) is still uncertain; this may vary among different vascular preparations.

The relaxations caused by ADP and ATP almost disappeared after removal of the endothelium and thus an involvement of the latter in the inhibitory response of isolated blood vessels to these nucleotides must be considered. Since the decreases in tension caused by adenosine and AMP were unaffected by the procedure, an explanation could be that, as is presumably the case for the prejunctional inhibitory effect of ATP (Verhaeghe, Vanhoutte & Shepherd, 1977; De Mey, Burnstock & Vanhoutte 1979), ATP (and ADP) must be degraded to adenosine (or AMP) by the endothelial cells in order to exert their inhibitory effect on the vascular smooth muscle cells of the media, but that the latter lack the enzymes necessary for this degradation. This interpretation is hard to reconcile with: (1) the findings that a concentration of theophylline which abolishes the relaxations caused by adenosine does not affect the inhibition evoked by ATP, and (2) the observation that ATP but not adenosine

causes relaxation of preparations made to contract with K^+. Thus, one is forced to conclude that, as is the case for ACh, ATP (and ADP) must act directly on the endothelial cells, which then in turn generate the trigger for relaxation of the deeper smooth muscle cells. The absence of effect of theophylline on the response to ATP, together with the independence of the effects of adenosine on the presence of the endothelium, indicates that the endothelial event involved is not due to activation of P_1 purinergic receptors (Burnstock, 1976, 1978). By contrast, activation of such P_1 receptors on the arterial smooth muscle cells provides a likely explanation for the inhibition of the contractile process caused by adenosine (and AMP).

REFERENCES

BAKHLE, Y. S. & VANE, J. R. (1974). Pharmacokinetic function of the pulmonary circulation. *Physiol. Rev.* **54**, 1007–1045.

BEVAN, J. A. & DUCKLES, S. P. (1975). Evidence for alpha-adrenergic receptors on intimal endothelium. *Blood Vessels* **12**, 307–310.

BUONASSISI, V. & VENTER, J. C. (1976). Hormone and neurotransmitter receptors in an established vascular endothelial cell line. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1612–1616.

BURNSTOCK, G. (1976). Purinergic 'receptors'. *J. theor. Biol.* **62**, 491–503.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: a Multidisciplinary Approach*, ed. STRAUB, R. W. & BOLIS, L., pp. 107–118. New York: Raven Press.

CAPLAN, B. A., GERRITY, R. G. & SCHWARTZ, C. J. (1974). Endothelial cell morphology in focal areas of *in vivo* Evans Blue uptake in the young pig aorta. I. Quantitative light microscopic findings. *Exp. & mol. Pathol.* **21**, 102–117.

DE MEY, J., BURNSTOCK, G. & VANHOUTTE, P. M. (1979). Modulation of the evoked release of noradrenaline in canine saphenous vein via presynaptic receptors for adenosine but not ATP. *Eur. J. Pharmacol.* **55**, 401–405.

DE MEY, J. G. VANHOUTTE, P. M. (1980*a*). Interaction between Na^+, K^+ exchanges and the direct inhibitory effect of acetylcholine on canine femoral arteries. *Circulation Res.* **46**, 826–836.

DE MEY, J. & VANHOUTTE, P. M. (1980*b*). Effect of removal and substitution of potassium ions on the adrenergic and cholinergic reactivity in canine femoral artery. *Eur. J. Pharmacol.* **67**, 159–164.

FURCHGOTT, R. G. & ZAWADZKI, J. (1980). Acetylcholine relaxes arterial smooth muscle by releasing a relaxing substance from endothelial cells. *Fedn Proc.* **39**, 581.

GILLIS, C. N. (1980). Metabolism of vasoactive hormones by pulmonary vascular endothelium: possible functional significance. In *Vascular Neuroeffector Mechanisms*, ed. BEVAN, J. A., GODFRAIND, T., MAXWELL, R. A. & VANHOUTTE, P. M. pp. 304–314. New York: Raven Press.

HADDY, F. J. (1978). The mechanism of potassium vasodilatation. In *Mechanisms of Vasodilatation*, ed. VANHOUTTE, P. M. & LEUSEN, I., pp. 200–205. Basel: S. Karger.

HENDERSON, R. M. (1975). Types of cell contacts in arterial smooth muscle. *Experientia* **31**, 103–105.

MONCADA, S. & VANE, J. R. (1978). Prostacyclin (PGI_2), the vascular wall and vasodilatation. In *Mechanisms of Vasodilatation*, ed. VANHOUTTE, P. M. & LEUSEN, I., pp. 107–121. Basel: S. Karger.

PASCUAL, R. & BEVAN, J. A. (1980). Evidence that changes in vascular tone may be initiated from the intima of the rabbit aorta. In *Vascular Neuroeffector Mechanisms*, ed. BEVAN, J. A. GODFRAIND, T., MAXWELL, R. A. & VANHOUTTE, P. M., pp. 300–304. New York: Raven Press.

RHODIN, J. A. G. (1967). The ultrastructure of mammalian arterioles and precapillary sphincters. *J. Ultrastruct. Res.* **18**, 181–223.

SHEPHERD, J. T. & VANHOUTTE, P. M. (1979). The human cardiovascular system, facts and concepts, pp. 1–351. New York: Raven Press.

VANHOUTTE, P. M. (1974). Inhibition by acetylcholine of adrenergic neurotransmission in vascular smooth muscle. *Circulation Res.* **34**, 317–326.

VENTER, J. C., BUONASSISI, V., BEVAN, S., HEINEMANN, S. & BEVAN, J. A. (1975). Hormone and neurotransmitter receptors on intimal endothelium. *Blood Vessels* **12**, 381.

VERHAEGHE, R. H., VANHOUTTE, P. M. & SHEPHERD, J. T. (1977). Inhibition of sympathetic neurotransmission in canine blood vessels by adenosine and adenine nucleotides. *Circulation Res.* **40**, 208–215.

WEBB, R. C. & BOHR, D. F. (1978). Potassium-induced relaxation as an indicator of Na^+, K^+ ATPase activity in vascular smooth muscle. *Blood Vessels* **15**, 198–207.