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Electrophysiological analysis of neurogenic vasodilatation in the isolated lingual artery of the rabbit

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1 The nature of neurogenic vasodilatation was investigated in isolated segments of rabbit lingual artery. In separate experiments membrane responses to nerve stimulation were studied by use of microelectrodes.

2 In the presence of guanethidine to block constrictor responses and noradrenaline to induce tone, field stimulation with trains of pulses (8 Hz for 0.5 to 4 s) produced vasodilatation. Atropine (10^{-6} M) reduced the relaxations to about 50% of the control values while the induced vasodilatations were potentiated by physostigmine. Tetrodotoxin (TTX, 10^{-7} M) blocked all nerve-evoked responses. These data suggest that there is a cholinergic and a non-cholinergic component of the vasodilatation produced by nerve stimulation in the rabbit lingual artery.

3 Single stimuli did not evoke electrophysiological responses. With parameters similar to those used in the mechanical studies, periarterial stimulation in the presence of guanethidine evoked membrane hyperpolarizations which achieved amplitudes of up to 11 mV. The ionophoretic application of acetylcholine (ACh) produced hyperpolarization.

4 The inhibitory junction potentials (i.j.ps) but not the ionophoretic-induced responses were blocked by TTX. The nerve-evoked and the ACh-induced hyperpolarizations were potentiated by physostigmine (5×10^{-7} M) and totally blocked by atropine (10^{-7} M).

5 I.j.ps and hyperpolarization to ionophoresis of ACh were recorded from arteries in which the endothelium had been removed by mechanical rubbing. Mechanical relaxation to field stimulation and ACh was observed in preparations without endothelium.

6 These data suggest that the cholinergic component of the neurogenic vasodilatation in the rabbit lingual artery is accompanied by hyperpolarization. The non-cholinergic component does not appear to possess an electrophysiological correlate. In addition, it seems that the action of nerve-released ACh is mediated by muscarinic receptors which are situated directly on the vascular smooth muscle cells.

Introduction

Neurogenic, cholinergic vasodilatation has been described in a number of vascular beds. For instance, stimulation of the sympathetic neural outflow to skeletal muscles of the cat and dog can evoke an atropine-sensitive vasodilatation, which is particularly evident when constrictor responses have been inhibited or in the presence of physostigmine (Bülbring & Burn, 1935). Neurogenic vasodilator responses that are attenuated by atropine and that probably are parasympathetic in origin have been noted in the cat cephalic circulation, specifically in blood vessels supplying the salivary glands (Lundberg, 1981), nasal mucosa (Eccles & Wilson, 1974), brain (Chorobski & Penfield, 1932, Bevan *et al.*, 1982), and tongue (Lundberg *et al.*, 1982; Bevan *et al.*, 1982). Such vascular responses have also been demonstrated in the guinea-pig uterine artery (Bell, 1968).

The objective of the present study was to determine if there are membrane electrophysiological events associated with neurogenic dilatation of the rabbit lingual artery, a blood vessel that supplies primarily the skeletal muscle of the tongue, but also mucous membranes and accessory salivary tissue in the posterior part of the tongue. A preliminary account of

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these findings has been presented (Brayden & Large, 1986).

Methods

Male rabbits weighing 1–2 kg were killed by cervical dislocation and the tongue was rapidly removed. Segments of proximal lingual artery having internal diameters of 0.5–0.8 mm were dissected from the tongue and placed in Krebs bicarbonate buffer of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.

For mechanical studies, two tungsten wires (diam.: 25 μm) were passed through the lumen of a 3.0 mm long arterial segment. One wire was attached to a stationary support and the other to a force transducer (Grass FT03) for isometric tension recording. The tissue was immersed in an organ bath (25 ml) containing warmed (34°C) Krebs bicarbonate solution which was gassed with 5% CO_2 in O_2 to maintain a pH of 7.4. Preparations were then given a pre-load of 0.5 g which in preliminary experiments was shown to be optimal for force development to field stimulation and exogenous noradrenaline. Periarterial nerves were activated by field stimulation with parallel silver wire electrodes placed on either side of the blood vessel. Square wave electrical pulses (10–30 V, 8 Hz, 0.3 ms duration) of various train durations were delivered by a Grass stimulator. In most experiments the adrenergic neurone blocker guanethidine (5×10^{-6}–10^{-5} M) was added to block neurogenic constrictor responses and 30 min later, tone was induced by addition of noradrenaline (10^{-7} M). The concentrations (5×10^{-6}–10^{-5} M) of guanethidine are larger than normally used to block transmitter release from sympathetic nerves. However with lower concentrations of guanethidine (10^{-6} M) nerve stimulation produced small contractions which produced artefacts with electrical recording.

In some preparations, endothelial cells were destroyed by briefly rubbing the luminal surface of the artery with a small metal dissecting pin. Removal of the endothelial cells was verified histologically (Poole *et al.*, 1958) at the end of these experiments.

Electrical recording

For electrophysiological measurements, arterial segments were pinned to a layer of Sylgard in the bottom of a perspex chamber. The tissue was superfused continuously with Krebs solution at a rate of 1–2 ml per min (the bath volume was approximately 0.5 ml). Membrane potentials were recorded from the adventitial surface with intracellular microelectrodes filled with 0.5 M KCl with resistances of 100–200 MΩ. Acetylcholine (ACh) was applied by ionophoresis using a constant current pump from similar micropipettes filled with 0.1–0.5 M ACh. The ionophoretic electrode was placed within 100 μm of the recording electrode. Nerve evoked responses were obtained by periarterial stimulation with the same parameters used in the mechanical experiments and the recording electrode was placed within 0.5–2.0 mm of the stimulating electrodes. Most electrophysiological experiments were carried out in the presence of guanethidine (5×10^{-6}–10^{-5} M).

Drugs

Drugs used were: noradrenaline bitartrate (Sigma); physostigmine sulphate (Sigma); guanethidine sulphate (Ciba); acetylcholine chloride (Sigma); atropine sulphate (Sigma); tetrodotoxin (Sigma).

Results

Mechanical responses

In normal Krebs solution, the rabbit lingual artery contracted during field stimulation (data not shown) and this response was abolished by guanethidine. Thirty min after exposure to guanethidine (5×10^{-6}–10^{-5} M) and in the presence of active tone induced by the addition of noradrenaline (10^{-7} M), arterial segments relaxed when perivascular nerves were stimulated (Figure 1a–c). Single electrical pulses elicited no response. However, trains of pulses lasting from 0.5–8 s at a frequency of 8 Hz induced graded relaxation of tone (Table 1) with an average time to the peak of the response of about 8 s. After exposure to atropine (10^{-7} M) for 5 min the relaxations were attenuated but not abolished (Figure 1a). Increasing the concentration of atropine to 10^{-6} M had no further inhibitory effect on the relaxation which accounted for about 50% of the total response following 2 s or 4 s of stimulation. After 15 min exposure to physostigmine (5×10^{-7} M) the overall response was greatly potentiated (Figure 1b). Tetrodotoxin (10^{-7} M) abolished the relaxations (Figure 1c).

Membrane responses

In normal Krebs solution the resting membrane potential was stable and had a value of -61.1 ± 1.2 mV (mean ± s.e.mean of 24 cells). In these conditions periarterial stimulation evoked excitatory junction potentials (e.j.ps) with a time to peak of about 100 ms. These responses were not reduced by the α-adrenoceptor blocking agent phentolamine (10^{-6} M) but were abolished in 10^{-7}–5×10^{-7} M TTX. If the frequency of stimulation was sufficiently high

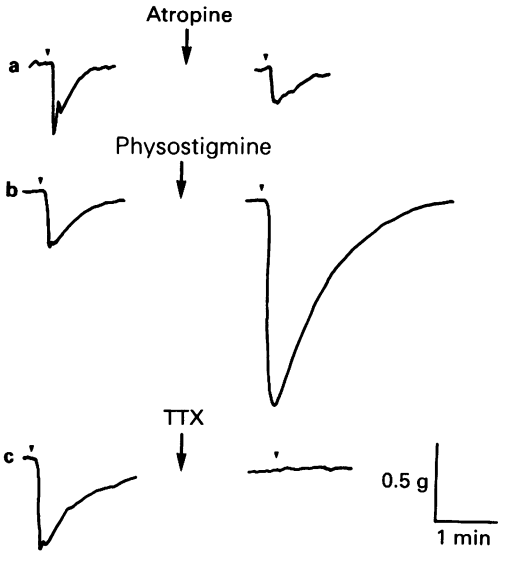

Figure 1 Effects of various drugs on vasodilatation evoked by activation of periarterial nerves in the rabbit lingual artery. Small arrows indicate the start of stimulation (1 second trains, 8 Hz). Control responses are shown on the left. Tissues were exposed to (a) atropine 10^{-7} M for 10 min, (b) physostigmine 5×10^{-7} M for 15 min, or (c) tetrodotoxin 10^{-7} M for 10 min before drug-effects were tested. Noradrenaline 10^{-7} M and guanethidine 10^{-5} M present throughout. Levels of active tone were 1.4 g (a), 1.7 g (b) and 1.2 g (c).

the e.j.ps summated and triggered an action potential and therefore the e.j.ps recorded in the rabbit lingual artery resemble the junction potentials recorded in many muscular arterial preparations (e.g. Surprenant, 1980). In the presence of 5×10^{-6}–10^{-5} M guanethidine the membrane potential was depolarized by 10 mV to a mean value of -51.0 ± 0.4 mV (mean ± s.e.mean of 28 cells). In the presence of guanethidine a single periarterial stimulus evoked no membrane response but several pulses delivered at 8 Hz produced hyperpolarization of the membrane and typical records are shown in Figure 2a–c. As with the mechanical responses at least four stimuli were required to produce hyperpolarization and the amplitude of the response increased with a greater number of applied pulses (Figure 2a–c). The maximum response (11 mV) was achieved with stimulation at 8 Hz for 4–8 s. This hyperpolarization presumably represents the summed activity of individual stimuli but we will refer to this response as an inhibitory junction potential (i.j.p.). The time to peak of the i.j.p. evoked by stimulation at 8 Hz for 2 s was 5.8 ± 0.4 s (mean ± s.e.mean in 21 cells, Table 2). The ionophoretic application of ACh also hyperpolarized the membrane (Figure 2d–f). These responses were dependent on the charge passed through the ionophoretic electrode and a maximum amplitude of 10 mV was obtained. These responses were characterized by a delay between the start of the ionophoretic pulse and the onset of the hyperpolarization. This latency was independent of pulse width and the values for hyperpolarizations evoked by ionophoretic application of ACh using pulse widths of 0.1–0.5 s have been pooled. The latency and time to peak of the ACh-induced hyperpolarizations were respectively 1.2 ± 0.1 s and 3.5 ± 0.1 s (Table 2). It should be noted that the rise time (and therefore the total time to peak) was greater with long pulse widths and the total time to peak value is included for the sake of completeness. Moreover it should be stressed that the values for the i.j.p. in Table 2 were measured from responses evoked by nerve stimulation for 2 seconds. It was not possible to observe if the i.j.p. was preceded by a latency because the early part of these records was obscured by stimulation artefacts.

The electrophysiological experiments were normally carried out in the absence of noradrenaline because the contraction produced by noradrenaline induced instability of the electrode impalement. However, a few experiments were carried out in the presence of

Table 1 Percentage relaxation of noradrenaline-induced tone and time to peak of neurogenic vasodilator responses

	Stimulus duration (8 Hz)			
	0.5 s	1.0 s	2.0 s	4.0 s
% relaxation				
Control	24 ± 7	42 ± 3	48 ± 3	59 ± 2
Plus atropine (10^{-7} M)	4 ± 2	11 ± 2	21 ± 3	29 ± 3
Time to peak (s)				
Control	—	7.2 ± 0.4	8.2 ± 0.5	8.3 ± 0.3

The figures are the mean ± s.e.mean from 5 preparations.

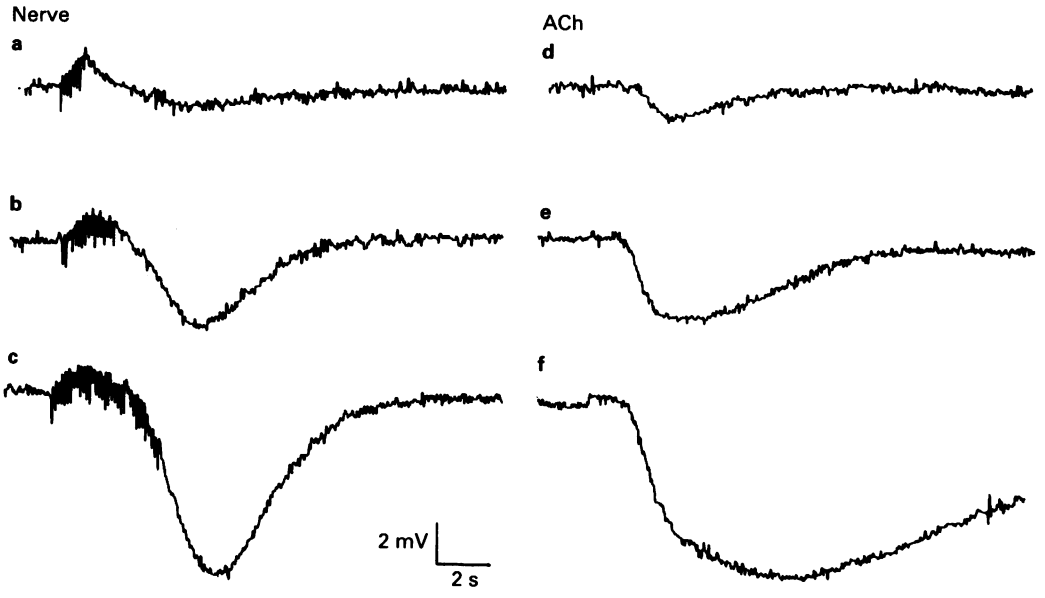

Figure 2 Hyperpolarizations evoked in the presence of guanethidine 10^{-5} M by nerve stimulation or ionophoresis of acetylcholine (ACh). Responses (a), (b) and (c) were produced by nerve stimulation at 30 V and 8 Hz for 1, 2 and 4 s respectively. Resting membrane potential (E_M): − 52 mV. (d), (e) and (f) were produced in another cell by ionophoresis of ACh (32 nA) for 50, 100 and 1000 ms respectively E_M: − 49 mV. The beginning of the ionophoretic pulses is indicated by a small upward deflexion in trace (f) which precedes the hyperpolarization.

10^{-7} M noradrenaline. In these conditions the membrane potential was depolarized to between − 40 and − 45 mV and nerve stimulation evoked hyperpolarizing responses of similar time course to the responses observed in the absence of noradrenaline. Moreover these responses were blocked by 10^{-7} M atropine (see later).

Figure 3 illustrates that in the presence of 5×10^{-7} M TTX the i.j.p. was abolished whereas a hyperpolarization of similar amplitude to ionophoretically-applied ACh was unaffected.

Pharmacology of the membrane responses

Since from the mechanical studies it appeared that the nerve-evoked vasodilatation consisted of a cholinergic and a non-cholinergic component the pharmacology of the membrane responses was investigated. Figure 4 illustrates the effect of the anticholinesterase drug physostigmine. After the addition of 5×10^{-7} M physostigmine to the bathing solution the amplitude of the i.j.p. increased within 5 min and the maximal effect was obtained in about 30 min (Figure 4a,b). In addition to potentiating the responses observed with submaximal stimulation, inactivation of cholinesterase increased the maximum amplitude of the hyperpolarization to 16 mV. In the presence of physostigmine the time to peak of the i.j.p. was 5.63 ± 0.34 s (Table 2), similar to the value obtained in the absence of physostigmine. In contrast, the total duration of the nerve-evoked hyperpolarization increased from a control value of 11.0 ± 0.1 s ($n = 13$) to 18.6 ± 1.6 s ($n = 15$) in the presence of physostigmine when using stimulation parameters of 8 Hz for 2 s.

Physostigmine produced similar effects on the hyperpolarizations induced by ionophoresis of ACh. The amplitude of the response was increased (Figure 4c,d) and the total duration of the hyperpolarization evoked by a 0.5 s pulse of ACh was increased from 13.4 ± 0.92 s ($n = 11$) to 46.2 ± 5.04 s ($n = 12$). In the presence of physostigmine the latency of the hyperpolarization had not altered (1.2 s in both Krebs and physostigmine, Table 2) but the time to peak increased (Figure 4c,d, Table 2) and the decay of the ionophoretic response was greatly prolonged.

The addition of low concentrations (10^{-7} M) of the muscarinic receptor antagonist atropine abolished the hyperpolarization evoked either by nerve stimulation or by ionophoresis of ACh (Figure 5).

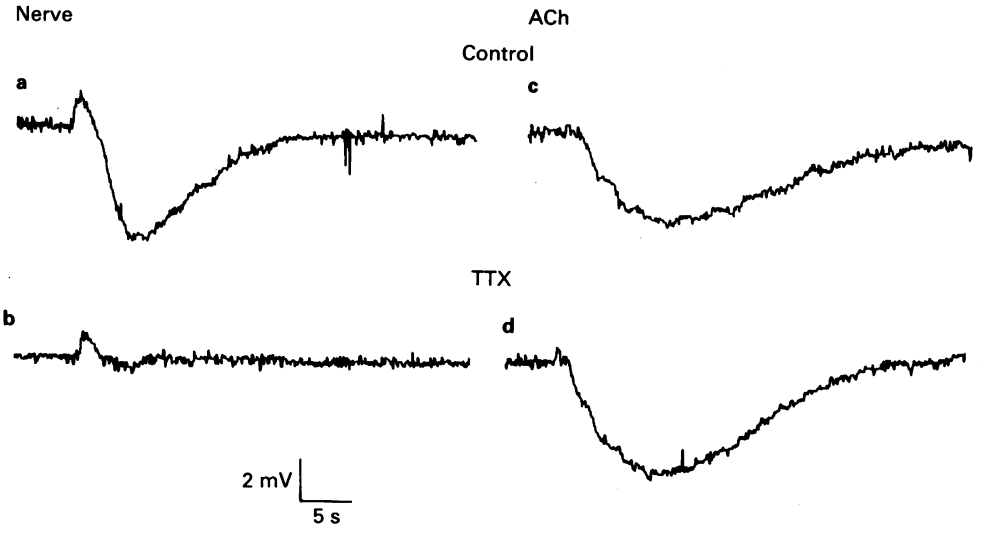

Figure 3 The effect of tetrodotoxin on the hyperpolarization produced by nerve stimulation and acetylcholine (ACh) in the same cell. Nerve responses (a) and (b) evoked by stimulation at 30 V and 8 Hz for 1 s and (c) and (d) were produced by ionophoresis of ACh (32 nA for 0.5 s). (a) and (c) are control responses and (b) and (d) were recorded 7 min after the addition of TTX 5×10^{-7} M. Physostigmine 5×10^{-7} M and guanethidine 10^{-5} M were present throughout, E_M: − 51 mV. In (a) and (b) the small artefactual upward deflection near the beginning of the trace indicates the start of nerve stimulation.

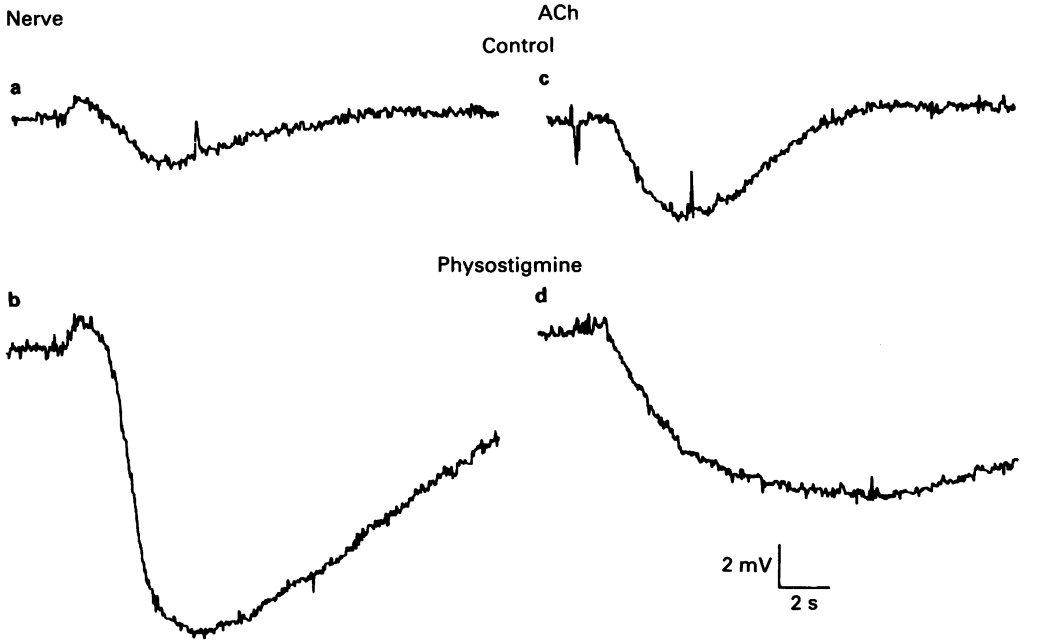

Figure 4 The effect of physostigmine on the responses to nerve stimulation and acetylcholine (ACh): (a) and (b) were produced by stimulation at 8 Hz for 0.5 s, (c) and (d) were evoked by ionophoresis of ACh (32 nA for 0.5 s). (a) and (c) are control responses and (b) and (d) were recorded 32 and 18 min respectively after the addition of physostigmine 5×10^{-7} M. E_M for (a) and (b): − 48 mV. E_M for (c) and (d): − 52 mV. Guanethidine 10^{-5} M present throughout.

Table 2 Time course of hyperpolarizations produced in the presence of guanethidine 10^{-5} M by nerve stimulation or ionophoresis of acetylcholine

	Amplitude (mV)	*Latency* (s)	*Total time to peak* (s)
Control			
Ionophoresis of ACh* ($n = 25$)	3.4 ± 0.4	1.2 ± 0.1	3.5 ± 0.1
i.j.p.† ($n = 21$)	6.7 ± 0.8	—	5.8 ± 0.4
Physostigmine 5×10^{-7} M			
Ionophoresis of ACh* ($n = 18$)	5.8 ± 0.9	1.2 ± 0.1	7.5 ± 0.5
i.j.p.† ($n = 17$)	11.7 ± 1.6	—	5.6 ± 0.3

*Parameters of ionophoresis were: pulse amplitude, 32 nA; pulse width, 0.1–0.5 s
†I.j.p. evoked by stimulation at 8 Hz for 2 s.
The i.j.p. latency was not estimated as stimulation artefacts obscured the beginning of the records.

Membrane responses in preparations without endothelium

We carried out experiments to see if the responses to nerve stimulation and ACh were altered in tissues in which the endothelium had been removed. The resting membrane potential in the smooth muscle cells from preparations without endothelium was −51.1 ± 1.4 mV ($n = 12$) in the presence of 10^{-5} M guanethidine. In these tissues, nerve stimulation evoked i.j.ps of similar amplitude to those recorded in preparations possessing an intact endothelium (e.g. Figure 6a,b). Thus in preparations without endothelium stimulation at 8 Hz for 2 s produced a mean i.j.p. amplitude of

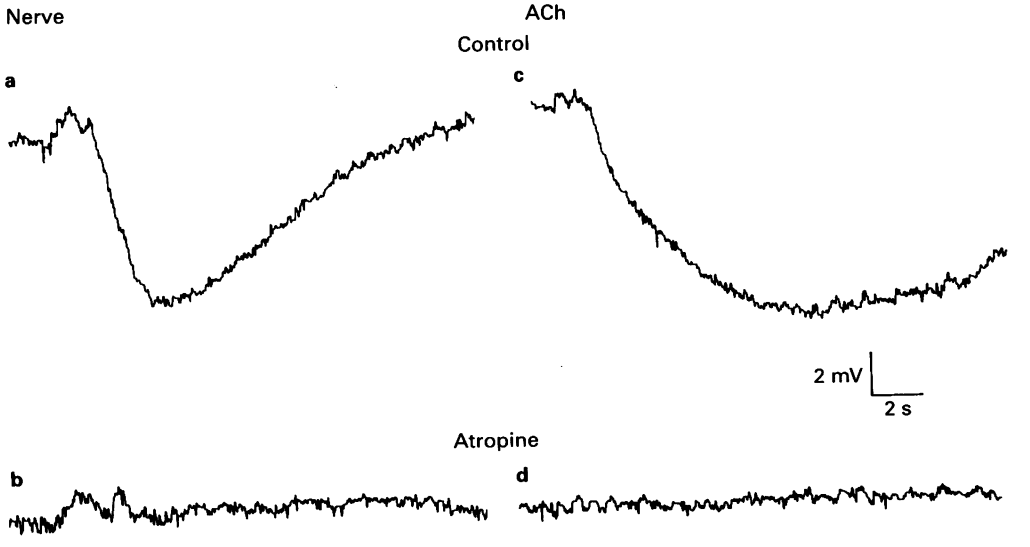

Figure 5 The effect of atropine on the nerve- and acetylcholine (ACh)-induced hyperpolarizations: (a) and (b) evoked by stimulation at 8 Hz for 1 s and (c) and (d) were produced by ionophoresis of ACh (32 nA for 0.5 s). (a) and (c) are control hyperpolarizations and (b) and (d) were recorded 8 min after the addition of atropine 10^{-7} M. Physostigmine 5×10^{-7} M and guanethidine 10^{-5} M present throughout, E_M: −54 mV.

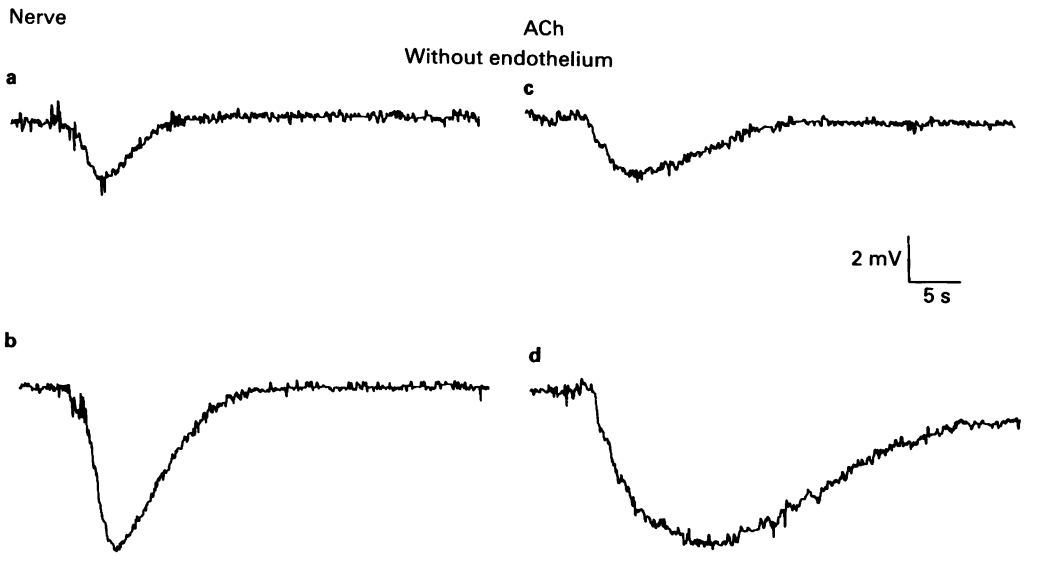

Figure 6 Responses to nerve stimulation or acetylcholine (ACh) in an artery without endothelium: (a) and (b) were evoked by stimulation at 8 Hz for 1 and 4 s respectively, (c) and (d) were produced by ionophoresis of ACh (32 nA) for 100 and 500 ms respectively. Physostigmine 5×10^{-7} M and guanethidine 10^{-5} M present throughout, E_M: -52 mV.

7.0 ± 0.3 mV ($n = 7$) compared to a control value of 7.8 ± 0.3 mV ($n = 9$). Also ionophoresis of ACh hyperpolarized the membrane (Figure 6c,d) in preparations without endothelium and these responses were also of similar amplitude to those observed in control tissues. It should be noted that atropine-sensitive vasodilatation to ACh and field stimulation persisted in preparations without endothelium.

Discussion

Despite the large number of tissues in which neurogenic, cholinergic vasodilatation has been observed, this is the first report of the occurrence of an associated vascular smooth muscle cell membrane hyperpolarization.

Only one other attempt to measure the electrophysiological correlates of neurogenic cholinergic vasodilatation has been described. Bell (1969) observed that the muscarinic antagonist hyoscine attenuated nerve-mediated vasodilator responses of perfused uterine arteries taken from pregnant guinea-pigs. Modest hyperpolarizations (5–6 mV) occurred in some preparations in response to periarterial nerve-stimulation in this preparation (Bell, 1969). However, these changes in membrane potential were not affected by hyoscine and were not mimicked by exogenous acetylcholine. We have no explanation for the differences between our observations and those in Bell's study. We can only suggest that such discrepancies may relate to species-difference or differences in the tissues supplied by the arteries, that is, uterine smooth muscle versus primarily skeletal muscle. Additional studies of this phenomenon in arteries supplying other tissues may shed some light on this question.

The inhibitory mechanical responses of the rabbit lingual artery consisted of two components. One was atropine-sensitive and accounted for about 50% of the amplitude of the dilatation but the other was unaffected by concentrations of atropine of up to 10^{-6} M. This type of mixed dilator response is not unusual; it has been noted in a number of different tissues including the cat salivary gland (Lundberg, 1981), nasal mucosa (Eccles & Wilson, 1974), tongue (Bevan *et al.*, 1982; Lundberg *et al.*, 1982) and the dog hind limb (Brody & Shaffer, 1970).

In the present study, nerve stimulation produced membrane hyperpolarization which was blocked by atropine. Therefore the cholinergic component of vasodilatation in the isolated rabbit lingual artery possesses an electrophysiological correlate. This does not seem to be so for the non-cholinergic component of vasodilatation as no membrane response to nerve stimulation was recorded in the presence of atropine. In these conditions vasodilatations to nerve stimula-

tion were observed. However since electrical and mechanical recordings were made in separate experiments it is not possible to conclude with certainty that the non-cholinergic component of vasodilatation in the rabbit lingual artery is mediated by a voltage-independent mechanism. The identity of the non-cholinergic inhibitory transmitter in the rabbit lingual artery is not known and our studies provide no evidence in support of any particular transmitter. Vasoactive intestinal polypeptide (VIP) has been suggested as a likely dilator transmitter candidate in a number of other blood vessels including the lingual artery of the cat (Lundberg *et al.*, 1982; Bevan *et al.*, 1984) and recent experiments have demonstrated that VIP does not exert its inhibitory effects via changes in membrane potential in the rabbit mesenteric artery (Itoh *et al.*, 1985). However, identification of the substance mediating the non-cholinergic responses in the rabbit lingual artery clearly awaits further study.

Vasodilatation induced by exogenous acetylcholine is known to depend on the presence of intact endothelial cells in many arterial preparations (see Furchgott, 1983). However, cholinergic vasodilatation that is neurogenic in origin does not seem to have a similar endothelial-cell dependence. In the present study several observations would argue for a direct effect of acetylcholine on muscarinic receptors located on the vascular smooth muscle cells. Membrane hyperpolarizations with latencies of about 1 s occurred in response to ionophoretic application of acetylcholine with very brief pulses (50 ms). It seems unlikely that, in 1 s, such small amounts of locally applied acetylcholine could traverse the vascular wall to exert effects on the endothelial cells which in turn release a substance that induces smooth muscle cell hyperpolarization. Indeed, such a mechanism of action was ruled out in our studies of arteries in which the endothelial cells had been destroyed. These observations are similar to those in a recent report on neurogenic cholinergic vasodilatation in the feline posterior auricular artery (Brayden & Bevan, 1985). Vasodilatation following stimulation of perivascular nerves, and in response to bath applied acetylcholine, occurred independently of endothelial-cells in that artery as well. Thus, this direct action of acetylcholine is not specific to a single arterial preparation or species and may turn out to be one of the better indices of a functional cholinergic dilator innervation of blood vessels.

An interesting question is whether the cholinergic-induced hyperpolarization *per se* causes the relaxation or whether the membrane response is some sort of incidental phenomenon. In the latter situation, relaxation may be effected by a voltage-independent mechanism such as an intracellular biochemical change. There is some evidence that the hyperpolarization is responsible for the relaxation. Firstly, the time to peak of the nerve-induced hyperpolarization was about 2 s less than the time to peak of the relaxation. Secondly, the parameters of stimulation for producing threshold and maximal responses were the same for both mechanical and electrical responses. The threshold membrane potential for causing contraction in most smooth muscle preparations is usually between -40 and -55 mV (Bolton & Large, 1986). The i.j.ps achieved amplitudes of up to 11 mV from a resting potential of -50 mV and this hyperpolarization should close at least some opened voltage-dependent calcium channels. Thus it is probable that stimulation of the cholinergic nerves can effect a voltage-dependent mechanism of relaxation in the rabbit lingual artery but an additional voltage-independent mechanism cannot be ruled out.

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