Br. J. Pharmac (1987), **92**, 657–664

Heterogeneous distribution of muscarinic receptors in the rabbit saphenous artery

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1 The properties of the muscarinic receptors in the rabbit saphenous artery were determined from electrical and mechanical responses of smooth muscle cells produced by acetylcholine (ACh). The inhibitory action of atropine and pirenzepine on the ACh-induced responses was also studied.

2 ACh produced a transient hyperpolarization of the membrane and inhibited the noradrenaline (NA)-induced contraction. These effects of ACh were apparent only when the endothelial cells were intact.

3 The ACh-induced transient hyperpolarization was antagonized by atropine or pirenzepine, with similar potencies (the ID_{50} values were about 2×10^{-8} M for both antagonists).

4 The ACh-induced inhibition of the contraction to NA was antagonized by atropine more preferentially than by pirenzepine (the ID_{50} values were 2×10^{-8} M for atropine and 10^{-6} M for pirenzepine).

5 The excitatory junction potential (e.j.p.) evoked by perivascular nerve stimulation was inhibited by ACh (above 10^{-8} M). The ACh-induced inhibition of the e.j.p. was antagonized by atropine more preferentially than by pirenzepine (the ID_{50} values were 3×10^{-8} M for atropine and 6×10^{-6} M for pirenzepine).

6 It is concluded that in the rabbit saphenous artery, two subtypes of muscarinic receptor (M_1 and M_2) are located on the endothelial cells. Stimulation of each subtype releases a different substance, i.e., a hyperpolarizing substance (M_1-subtype) or a relaxant substance (M_2-subtype). In prejunctional nerve terminals, the muscarinic receptors responsible for inhibiting the release of transmitter substances are of the M_2-subtype.

Introduction

Although vasomotor nerves are mainly adrenergic, there are some arteries which are innervated by cholinergic vasodilator nerves (Brayden & Large, 1986). However, muscarinic receptors are distributed widely in vascular tissues (Bevan *et al.*, 1980). Stimulation of muscarinic receptors located on noradrenergic nerve terminals by exogenously applied acetylcholine (ACh) inhibits the release of transmitter substances, as determined by measuring the release of incorporated [^{3}H]-noradrenaline during transmural nerve stimulation (Vanhoutte *et al.*, 1973; Vanhoutte, 1974; Fozard, 1979) or inhibition of the excitatory junction potential (e.j.p.) evoked by perivascular nerve stimulation (Kuriyama & Suzuki, 1981). Direct actions of ACh on vascular smooth muscle cells had been suggested from ACh-induced vasodilatation (Su, 1977) or ACh-induced hyperpolarization of the membrane (Kuriyama & Suzuki, 1978; Kitamura & Kuriyama, 1979; Takata, 1980; Brayden & Large, 1986). However, ACh-induced dilatation is mediated mainly by substances released from endothelial cells (Furchgott & Zawadzki, 1980; Furchgott, 1983). Also ACh-induced hyperpolarization is mainly due to substances released from the endothelial cells, and a direct action of muscarinic agonists on vascular smooth muscle cells results in either depolarization of the membrane (Bolton *et al.*, 1984) or an almost negligible effect (Komori & Suzuki, 1987). However, in the rabbit lingual artery, ACh hyperpolarizes directly the smooth muscle cells and the endothelial cells do not appear to be involved (Brayden & Large, 1986).

Muscarinic receptors are classified into M_1 and M_2 subtypes, from differences in responses to muscarinic agonists in the oesophageal sphincter muscles (Goyal & Rattan, 1978). Hammer *et al.* (1980) demonstrated, in receptor binding experiments, that pirenzepine has a higher affinity for muscarinic receptors in nervous tissue than for those in peripheral effector organs. The muscarinic receptors with a higher affinity for pirenzepine are classified as the M_1 subtype, while those

with a lower affinity for pirenzepine are of the M_2 subtype (Hammer & Giachetti, 1982). Based on the selectivity for pirenzepine, the muscarinic receptors in the sympathetic ganglia are classified as the M_1 subtype (Brown *et al.*, 1980), while those in ileal smooth muscle and the heart are of the M_2 subtype (Brown *et al.*, 1980; Fuder *et al.*, 1982; Barlow & Chan, 1982). In the guinea-pig enteric nervous system, activation of M_1- and M_2-receptors produces postsynaptic depolarization and presynaptic inhibition of transmitter release, respectively (North *et al.*, 1985). However, pre- and post-junctional muscarinic receptors in the guinea-pig ileum are pharmacologically identical (Szelenyi, 1982; Kilbinger *et al.*, 1984).

We investigated the effects of ACh and its inhibition by atropine and pirenzepine, on smooth muscle cells and on noradrenergic transmission in the rabbit saphenous artery, to clarify the properties of muscarinic receptors at vascular pre- and post-junctional membranes. The properties of the prejunctional muscarinic receptors were evaluated from the changes in amplitude of the e.j.p., and those of the postjunctional muscarinic receptors by recording changes in membrane potential and mechanical responses of the smooth muscle cells.

Methods

Young albino rabbits (2–3 months old) of either sex, weighing 1.8–2.5 kg, were anaesthetized with pentobarbitone (40 mg kg^{-1}, i.v.), and exsanguinated. Segments of the saphenous artery, 1–2 cm long, were dissected and the connective tissues removed in Krebs solution at room temperature.

The isolated segment of the saphenous artery (about 2 cm long) was mounted in an experimental chamber (the volume being about 2 ml), and superfused with warmed (35°C) Krebs solution at a flow rate of 2–3 ml min^{-1}. Perivascular nerves were stimulated by the point-stimulation method with current pulses of 0.02–0.05 ms duration and 20–50 V intensity. Arterial smooth muscle cells were impaled from the outer surface of the vessel with a glass capillary microelectrode filled with 3 M KCl (the resistance of the electrodes ranged between 50 and 80 MΩ). Electrical responses of the smooth muscle membrane were displayed on a pen-writing recorder (Nihon Kohden recticorder RJG 4024).

Helically cut strips of the artery (about 0.2 cm width, 2 cm, 0.15–0.2 mm thick) were suspended in a recording chamber and superfused with Krebs solution (35°C). The recording chamber was cylindrical (about 5 mm in diameter) with a volume of about 0.4 ml. Both ends of the tissue were connected with silk thread; one end was fixed at the bottom of the chamber and the other end to a mechano-transducer (Nihon Kohden, FD pick-up, TB612T) for isometric tension recording. The mechanical responses were displayed on a pen-writing recorder (National, VP-6521A).

The Krebs solution had the following ionic composition (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134, glucose 11.5. The solution was aerated with 95% O_2 and 5% CO_2 and the pH of the solution was kept at 7.3–7.4.

Drugs used were acetylcholine chloride, atropine sulphate, noradrenaline hydrochloride (Sigma), guanethidine sulphate (Tokyo Kasei), tetrodotoxin (Sankyo) and pirenzepine (Boehringer Ingelheim).

The results are expressed as the mean ± s.d. Statistical significances were determined by use of Student's t test and probabilities of less than 5% ($P < 0.05$) were considered significant.

Results

ACh-induced hyperpolarization of smooth muscle membrane

Smooth muscle cell membranes of the rabbit saphenous artery were electrically quiescent, and did not show any spontaneous activity. Application of ACh (10^{-5} M) produced a transient hyperpolarization of the membrane, the amplitude and duration being 4–6 mV and 1–4 min respectively.

Figure 1 shows the effects of atropine and pirenzepine on the ACh-induced hyperpolarization in the rabbit saphenous artery. ACh (10^{-5} M) was applied for about 1 min, because the peak amplitude of the ACh-induced hyperpolarization was reached within 1 min (the mean time required to reach peak hyperpolarization was 47.3 ± 8.0 s, $n = 10$). Pretreatment with atropine (10^{-8}–10^{-6} M) or pirenzepine (10^{-8}–10^{-6} M) for over 20 min did not change the resting membrane potential (Table 1). After pretreatment (for 20 min) with 10^{-6} M atropine or pirenzepine, the ACh-induced hyperpolarization was abolished.

The concentration-response relationship of the effects of atropine and pirenzepine pretreatment on the amplitude of 10^{-5} M ACh-induced hyperpolarization is shown in Figure 2. The amplitude of the ACh-induced hyperpolarization in the presence of atropine or pirenzepine is expressed as a % inhibition of the control value (i.e., $(V - V') \times V^{-1} \times 100$, where V and V′ are amplitudes of the ACh-induced hyperpolarization produced before and after application of antagonist, respectively). Both atropine and pirenzepine inhibited the ACh-induced hyperpolarization in concentrations above 10^{-8} M, and the relationship was much the same for both drugs. The calculated ID_{50} values (concentration of antagonist to inhibit the ACh-induced hyperpolarization to 50% of the control) were $2.2 \pm 0.8 \times 10^{-8}$ M ($n = 5$) for atropine and

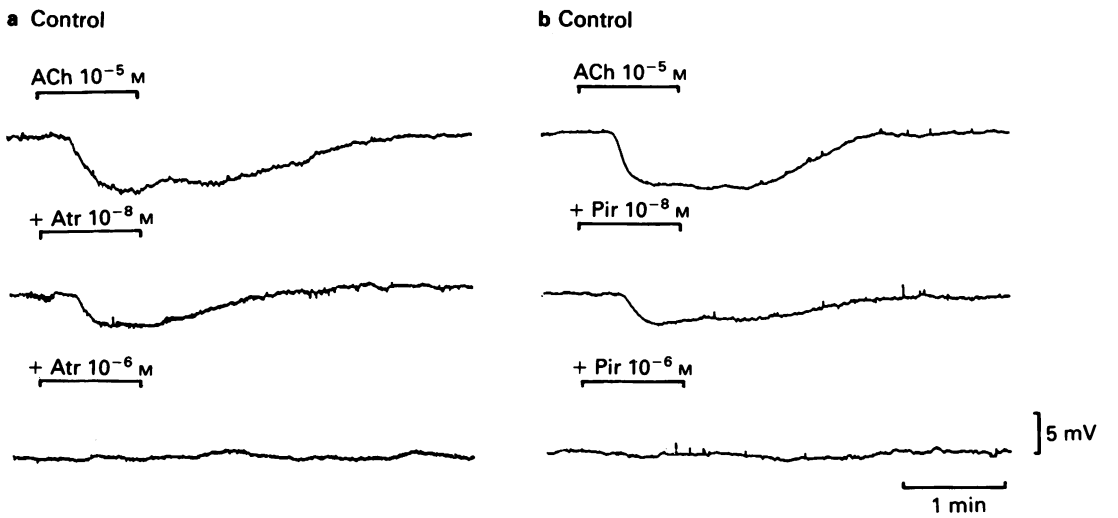

Figure 1 Effects of (a) atropine and (b) pirenzepine on the acetylcholine (ACh, 10^{-5} M)-induced hyperpolarization of smooth muscle cell membrane. ACh (10^{-5} M) was applied as indicated by the bar above each record, before (control) and after pretreatment (for 20 min) with atropine (Atr, 10^{-8} or 10^{-6} M) (a) or pirenzepine (Pir, 10^{-8} or 10^{-6} M) (b). (a) and (b) were recorded from different tissues.

$2.4 \pm 0.5 \times 10^{-8}$ M ($n = 5$) for pirenzepine. These two values were not statistically significant ($P > 0.05$).

Effects of atropine and pirenzepine on the ACh-induced relaxation

Application of ACh (above 10^{-7} M) inhibited the NA (10^{-6} M)-induced contraction in a concentration-dependent manner only when the endothelium was intact, thereby indicating that the relaxation was mainly due to a substance released from the endothelial cells in the rabbit saphenous artery. Figure 3 shows the effects of atropine and pirenzepine on the ACh (10^{-5} M)-induced relaxation. Atropine (above 10^{-9} M) inhibited the actions of ACh, and 10^{-6} M atropine completely blocked the ACh-induced inhibition of the NA-induced contraction. Pirenzepine (above 10^{-8} M) also antagonized the actions of ACh. Atropine was about 100 times more potent than pirenzepine in inhibiting the ACh-induced relaxation. The ID_{50} values were $2.2 \pm 0.9 \times 10^{-8}$ M ($n = 5$) and $1.4 \pm 0.6 \times 10^{-6}$ M ($n = 5$) for atropine and pirenzepine, respectively.

Table 1 Effects of drugs on the membrane potential of rabbit saphenous artery

	Membrane potential (mV)	
Control	-68.9 ± 1.6	(24)
ACh 10^{-5} M	-68.6 ± 1.8[a]	(15)
Atropine 10^{-6} M	-69.0 ± 1.6[a]	(13)
Pirenzepine 10^{-5}	-68.9 ± 1.3[a]	(14)
ACh 10^{-5} M + Atr 10^{-6} M	-69.6 ± 1.4[a]	(16)
ACh 10^{-5} M + Pir 10^{-5} M	-68.9 ± 1.5[a]	(15)
NA 10^{-6} M	-61.1 ± 2.3[b]	(15)
NA 10^{-6} M + Atr 10^{-6} M	-61.3 ± 2.3[c]	(15)
NA 10^{-6} M + Pir 10^{-5} M	-61.5 ± 2.0[c]	(13)

Data shown are mean ± s.d. of number of observations shown in parentheses. Membrane potentials were recorded during 5–30 min application of drugs, by successive impalements of the electrode into different cells.
[a]Not significantly different from the control ($P > 0.05$).
[b]Significantly different from the control ($P > 0.05$).
[c]Not significantly different from the NA-induced depolarization ($P > 0.05$).
ACh = acetylcholine, Atr = atropine, Pir = pirenzepine and NA = noradrenaline.

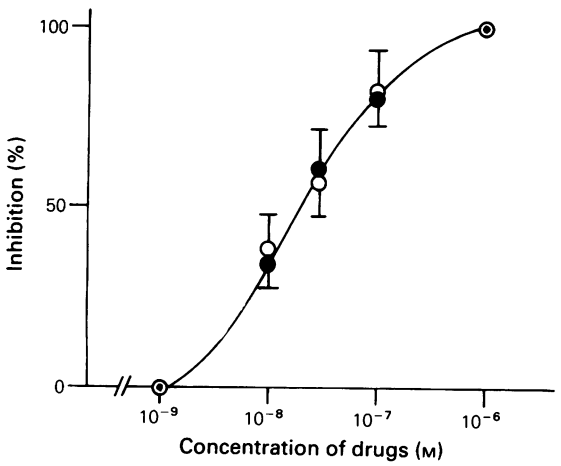

Figure 2 Concentration-response relationship of the effects of atropine and pirenzepine on the acetylcholine (ACh)-induced hyperpolarization. Vertical axis indicates $(V\text{-}V')\ V^{-1} \times 100\%$ where V and V' were amplitudes of the ACh (10^{-5} M)-induced hyperpolarization before and after application of atropine (○) or pirenzepine (●), respectively. Atropine or pirenzepine was applied for over 20 min before the application of ACh. Each point represents mean of $n = 5$ and vertical lines indicate s.d.

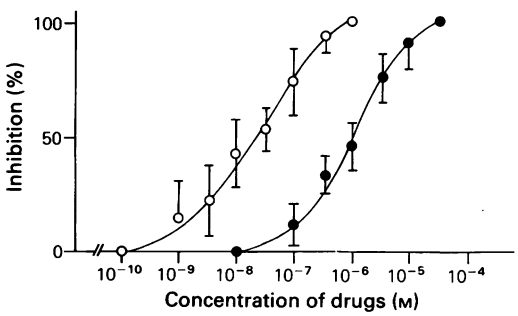

Figure 3 Effects of atropine and pirenzepine on the acetylcholine (ACh, 10^{-5} M)-induced inhibition of noradrenaline (NA, 10^{-6} M)-induced contractions. Vertical axis indicates $(V\text{-}V') \times V^{-1} \times 100\%$ where V and V' are amplitude of 10^{-5} M ACh-induced relaxation before and after application of atropine (○) or pirenzepine (●), respectively. Atropine or pirenzepine was applied for over 20 min before the addition of ACh. Each point represents mean of $n = 8$–10 and vertical lines indicate s.d.

Inhibition of e.j.p. by ACh

Figure 4 shows the effects of ACh (10^{-8} M–10^{-5} M) on the e.j.ps evoked by perivascular nerve stimulation (10 stimuli at 0.5 Hz frequency) in the rabbit saphenous artery. The e.j.p. was recorded after application of ACh for over 50 min, at a time when the transient

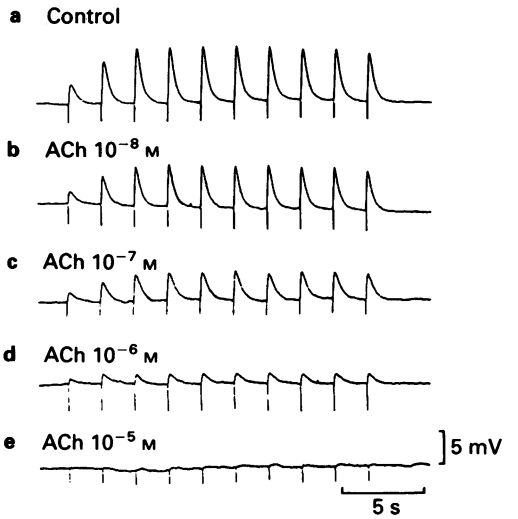

Figure 4 Effects of acetylcholine (ACh) on the e.j.p. evoked in the rabbit saphenous artery by perivascular nerve stimulation (0.03 ms in duration and 25 V intensity). A train of 10 stimuli at 0.5 Hz frequency was applied before (control) and after application of ACh (10^{-8}–10^{-5} M) for over 5 min. All the responses were recorded from a single cell.

hyperpolarization had ceased. ACh inhibited the amplitude of the e.j.p. in a concentration-dependent manner, and with 10^{-5} M ACh the e.j.ps were almost abolished. The inhibitory effects of ACh on the e.j.p. were reversible, and washing out of ACh for 5 min was sufficient for recovery.

Experiments were carried out to determine the effects of 10^{-5} M ACh on the e.j.ps evoked after pretreatment with atropine or pirenzepine. Atropine (above 10^{-8} M) antagonized the ACh-induced inhibition of the e.j.p., and with 10^{-6} M atropine, ACh did not cause any detectable change in the e.j.p. amplitude (Figure 5a). In similar experiments, the threshold concentration of pirenzepine required to antagonize the ACh-induced inhibition of the e.j.p. was 10^{-6} M, and 10^{-4} M pirenzepine completely blocked the actions of ACh on the e.j.p. (Figure 5B).

Figure 6 summarizes the antagonistic actions of atropine and pirenzepine on the ACh-induced inhibition of the e.j.ps. Atropine was more than 100 times more potent than pirenzepine in inhibiting the actions of ACh. The ID_{50} values were $3.0 \pm 0.8 \times 10^{-8}$ M ($n = 6$) and $6.1 \pm 1.4 \times 10^{-6}$ M ($n = 5$) for atropine and pirenzepine, respectively.

Effects of ACh, atropine and pirenzepine on the facilitation of e.j.ps

Attempts were made to estimate the effects of ACh,

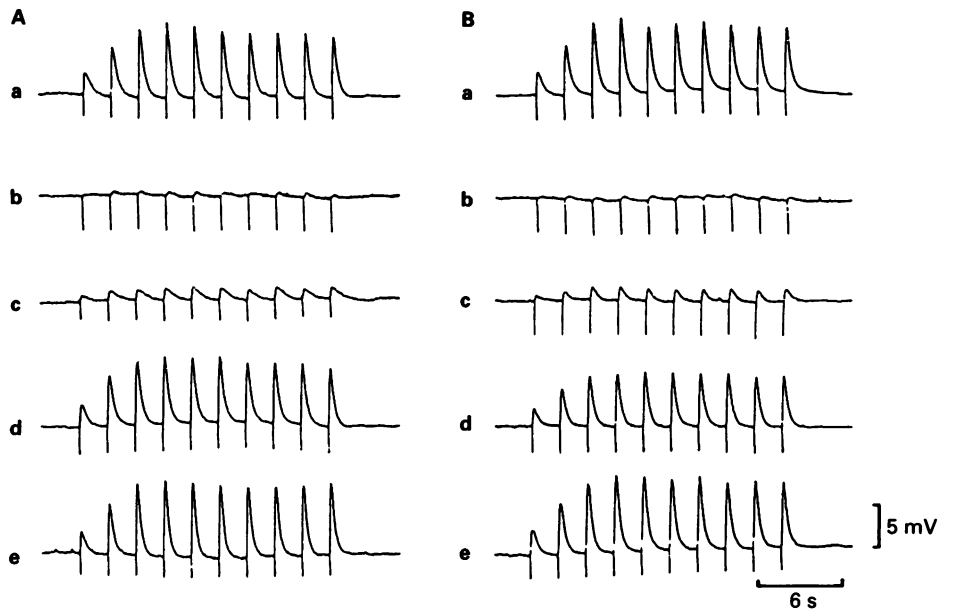

Figure 5 Effects of atropine and pirenzepine on the e.j.ps which were inhibited by acetylcholine (ACh, 10^{-5} M). A train of 10 pulses at a frequency of 0.5 Hz was applied. The tissues were pretreated with atropine (10^{-8}–10^{-6} M) (A) or pirenzepine (10^{-6}–10^{-4} M) (B) for 20 min, and then 10^{-5} M ACh was added. (A) and (B) were recorded from single cells in different tissues. (A), (a) Control, (b) ACh 10^{-5} M, (c) ACh plus atropine 10^{-8} M, (d) ACh plus atropine 10^{-7} M, (e) ACh plus atropine 10^{-6} M. (B), (a) Control, (b) ACh 10^{-5} M, (c) ACh plus pirenzepine 10^{-6} M, (d) ACh plus pirenzepine 10^{-5} M, (e) ACh plus pirenzepine 10^{-4} M.

atropine and pirenzepine on facilitation of e.j.ps by applying two stimuli at various intervals (1–5 s) (Figure 7). The amplitude of the second e.j.p. (V') was plotted as a function of $(V'-V) \times V^{-1}$ on a log scale against the interval of the two stimuli (V and V' were amplitudes of the first and the second e.j.ps, respectively; Mallart & Martin, 1967). The relationship was linear up to a 5 s interval. ACh (10^{-8} M) reduced the amplitude of e.j.p. to about 70% of the control. However, the facilitation process of the e.j.ps was not

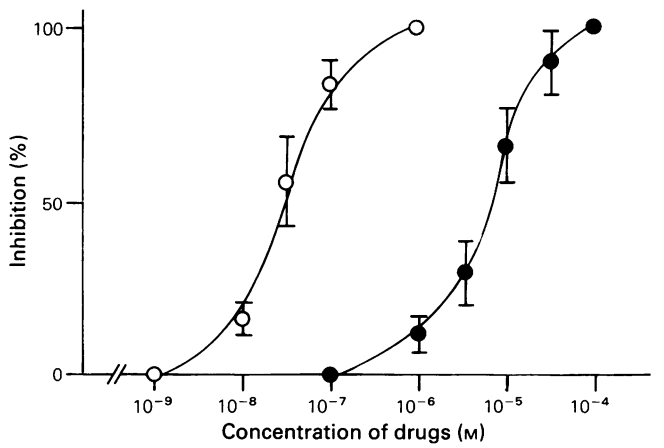

Figure 6 Concentration-response relationship of the effects of atropine (○) and pirenzepine (●) on the acetylcholine (ACh, 10^{-5} M)-induced inhibition of the e.j.p. in the rabbit saphenous artery. Vertical axis indicates (V_2-V_1) $V-V_1)^{-1} \times 100\%$ where V = control amplitude, V_1 = e.j.p. amplitude in the presence of ACh (10^{-5} M) and V_2 = e.j.p. amplitude in the presence of atropine or pirenzepine and ACh (10^{-5} M).

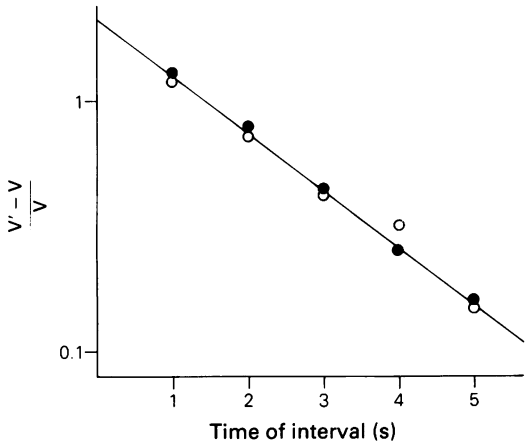

Figure 7 Effects of acetylcholine (ACh) on the facilitation process of the e.j.p. evoked by perivascular nerve stimulation (0.03 ms pulse duration, 30 V intensity). A pair of stimuli (1–5 s in interval) was applied, and the net increase in amplitude of the second e.j.p. (V') relative to that of the first (V) (i.e., $(V'-V)\,V^{-1}$ was plotted as a function of time between the two stimuli. Control (○) and ACh 10^{-8} M (●). The time constant of the facilitation of e.j.ps was calculated from the regression line in the figure as time decayed to 1/e.

changed. The calculated time constant of the facilitation of the e.j.p. (τf) remained unchanged after application of 10^{-8} M ACh (control, $\tau f = 2.11 \pm 0.2$ s, $n = 10$; in ACh, $\tau f = 2.07 \pm 0.13$ s, $n = 7$; $P > 0.05$). Atropine (10^{-6} M) or pirenzepine (10^{-5} M) blocked the ACh-induced inhibition of the e.j.p. amplitude without changing the facilitation. In the presence of atropine (10^{-6} M) or pirenzepine (10^{-5} M), 10^{-8} M ACh also had no effect on the τf (in atropine, $\tau f = 2.08 \pm 0.13$ s, $n = 5$; in pirenzepine, $\tau f = 2.01 \pm 0.16$ s, $n = 5$; $P > 0.05$).

The e.j.p. evoked in the rabbit saphenous artery decayed exponentially with a time constant of 260–280 ms (Holman & Surprenant, 1979). The potential plotted on a log scale against time decayed linearly, up to 500 ms. The time constant of the falling phase of the e.j.p. was calculated from the time when the potential decayed to 1/e. Application of ACh (10^{-8} M) inhibited the amplitude of the e.j.p. to about 70% of the control, with no change in the time constant of the decay of the potential (control, 268 ± 24 ms, $n = 10$; in ACh, 260 ± 20 ms, $n = 8$). Application of atropine (10^{-6} M) or pirenzepine (10^{-5} M) alone or together with 10^{-8} M ACh did not cause any detectable change in the time constant of the falling phase of the e.j.p. (control, 265 ± 18 ms, $n = 8$; in pirenzepine, 258 ± 10 ms, $n = 8$; in atropine, 264 ± 15 ms, $n = 9$).

Effects of noradrenaline on the membrane potential

In the rabbit saphenous artery, application of noradrenaline (NA, above 3×10^{-7} M) depolarized the smooth muscle membrane, in a concentration-dependent manner, and reached a maximum depolarized value of -58.7 ± 1.7 mV ($n = 13$) with 10^{-5} M NA. The NA (10^{-6} M)-induced depolarization was not affected by the additional application of atropine (10^{-6} M) or pirenzepine (10^{-5} M) (Table 1).

Discussion

Smooth muscle cells of the rabbit saphenous artery responded to ACh with both a transient hyperpolarization of the membrane and an inhibition of the NA-induced contraction. These actions of ACh could be seen only when the endothelial cells were intact. Removal of the endothelial cells by rubbing the internal lumen of the vessel caused no detectable change in the resting membrane potential or mechanical responses during application of ACh (Komori & Suzuki, 1987). Thus, in this artery, direct actions of ACh on the smooth muscle may be weak, and ACh acts on the smooth muscle cells indirectly by releasing hyperpolarizing and relaxing substances from the endothelial cells. The ACh-induced endothelium-derived substances which hyperpolarize the smooth muscle membrane, may be different from the endothelium-derived relaxing factor (EDRF, Furchgott, 1983), in that the former produces transient actions while the latter relaxes the muscle for a long time (10–20 min, Komori & Suzuki, 1987). The present experiments demonstrated that the ACh-induced hyperpolarization can be blocked by either atropine or pirenzepine, at similar concentrations. As pirenzepine, as well as atropine, has a high affinity for the M_1-subtype of receptor (Hammer *et al.*, 1980; Hammer & Giachetti, 1982), the muscarinic receptors involved in the release of the hyperpolarizing substance from the endothelial cells are mainly the M_1 subtype. Atropine was about 100 times more potent than pirenzepine in antagonizing the ACh-induced relaxation of the artery precontracted with 10^{-6} M NA. Therefore, the muscarinic receptors involved in the release of the relaxing substance from the endothelium are mainly the M_2 subtype. These results strongly suggest that two different substances are released from the endothelial cells, i.e. stimulation of M_1- or M_2-receptors located on the endothelium releases a hyperpolarizing or relaxing substance, respectively.

Stimulation of muscarinic receptors located on adrenergic nerve terminals by ACh inhibits the release of noradrenaline (NA, Vanhoutte *et al.*, 1981). We estimated the effects of ACh on noradrenergic transmission in the rabbit saphenous artery from changes in

amplitude of the e.j.p. evoked by perivascular nerve stimulation. An e.j.p. can be evoked in many muscular arteries including the rabbit saphenous artery (Kuriyama *et al.*, 1982). The e.j.p. is resistant to α-adrenoceptor antagonists, but can be blocked by guanethidine or chemical denervation of adrenergic nerves by 6-hydroxydopamine (Miyahara & Suzuki, 1985), thereby indicating that this potential may be generated by substances released from perivascular adrenergic nerves. By comparing the e.j.p. with the slow depolarization which may be generated by NA, the e.j.p. could be used as an indicator of the amount of NA released during perivascular nerve stimulation (Suzuki, 1984; Suzuki *et al.*, 1984). The present experiments demonstrated that in the rabbit saphenous artery ACh inhibited the amplitude of the e.j.p., as has been demonstrated in the guinea-pig mesenteric artery (Kuriyama & Suzuki, 1981). Both atropine and pirenzepine antagonized the ACh-induced inhibition of the e.j.ps. However, atropine was over 100 times more potent than pirenzepine in antagonizing the effect of ACh. The concentration of atropine required to antagonize the ACh-induced inhibition of the e.j.p. was comparable to that required to inhibit the ACh-induced transient hyperpolarization. This suggests that the muscarinic receptors involved in inhibiting the release of the transmitter substances are mainly of the M_2 subtype.

During application of ACh, the NA-induced depolarization of the membrane remained unchanged, i.e., ACh probably does not reduce the chemical sensitivity of the postjunctional membrane. The resting membrane potential, the membrane resistance estimated from the amplitude of electrotonic potential (Komori & Suzuki, 1987) and the time constant of the falling phase of the e.j.p. were not changed by ACh, suggesting that ACh did not change the biophysical properties of the smooth muscle membrane. Therefore, the ACh-induced inhibition of the e.j.p. may be due to a reduction in the amount of transmitter substance(s) which is responsible for generation of the e.j.ps. In the presence of ACh, the amplitude of the e.j.ps was inhibited with no change in the facilitation process. The facilitation of e.j.ps estimated by the method of Mallart & Martin (1967) may reflect an increase in the amount of residual Ca ions available for transmitter release in the nerve terminal (Katz & Miledi, 1968). Thus, in the rabbit saphenous artery, ACh reduces the amount of transmitter released but does not inhibit the rate of mobilization of Ca ions required for the exocytosis. The potency of ACh in inhibiting the e.j.p. was not different in tissues with endothelial cells from those without endothelial cells (Komori & Suzuki, 1987), suggesting that endothelium-derived substances are not involved in the ACh-induced inhibition of the e.j.ps.

It is concluded that in the rabbit saphenous artery, two subtypes of muscarinic receptor (M_1 and M_2) are located on the endothelial cells, and stimulation of the M_1 subtype releases a hyperpolarizing substance while that of the M_2 subtype releases a relaxing substance from these cells. The prejunctional muscarinic receptors responsible for inhibiting the transmitter release are of the M_2 subtype.

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(Received May 15, 1987.
Revised June 26, 1987.
Accepted July 15, 1987.)