Br. J. Pharmacol. (1988), **93**, 636–646

Autoregulation of acetylcholine release from vagus nerve terminals through activation of muscarinic receptors in the dog trachea

Yushi Ito & Takeshi Yoshitomi

Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

1 The effects of pirenzepine and gallamine on the membrane and contractile properties of smooth muscle cells and on excitatory neuro-effector transmission in the dog trachea were investigated by means of microelectrode, double sucrose gap and tension recording methods.

2 Pirenzepine (10^{-7} M) and gallamine (10^{-5} M) had no effect on the resting membrane potential or the input resistance of the smooth muscle cells.

3 Pirenzepine (10^{-10}–10^{-9} M) and gallamine (10^{-7} M) enhanced the amplitude of twitch contractions evoked by field stimulation in the combined presence of indomethacin (10^{-5} M) and propranolol (10^{-6} M). At higher concentrations pirenzepine (10^{-8} M) inhibited the twitch contractions in a dose-dependent manner. Both pirenzepine and gallamine in doses over 10^{-7} and 10^{-5} M, respectively, reduced muscle tone.

4 Pirenzepine (10^{-10}–10^{-9} M) and gallamine (10^{-7} M) enhanced the amplitude of excitatory junction potentials (e.j.ps) evoked by field stimulation (single or repetitive stimulation). However, a high concentration of pirenzepine (10^{-8} M) reduced the amplitude of e.j.ps. In parallel with its action on e.j.ps, pirenzepine (over 10^{-9} M) reduced the response of smooth muscle cells to acetylcholine (ACh), in a dose-dependent manner. Gallamine (5×10^{-5} M) markedly enhanced the amplitude of e.j.ps but also reduced the response of muscle cells to ACh.

5 ACh (10^{-10}–10^{-9} M) inhibited twitch contractions evoked by field stimulation, with a slight increase of resting tension.

6 Gallamine enhanced the summation of e.j.ps during repetitive field stimulation at a high frequency (20 Hz), but was without effect on the depression phenomena of e.j.ps observed during double stimulus experiments at different time intervals (5–60 s).

7 These results indicate that both pirenzepine and gallamine have dual actions on pre- and post-junctional muscarinic receptors in dog tracheal tissue. At low concentrations both agents potentiate excitatory neuro-effector transmission, presumably due to enhancement of release of ACh from vagal nerve terminals through blockade of a negative auto-regulatory process activated by endogenous ACh. At higher concentrations, these agents inhibit the response of smooth muscle cells to ACh through post-junctional muscarinic receptors and relaxation of the muscle tissue occurs.

Introduction

The existence of inhibitory neuronal muscarinic receptors has been demonstrated in central (Watson *et al.*, 1983; Raiteri *et al.*, 1982; 1984) and peripheral adrenergic (Starke, 1977, Fuder *et al.*, 1981; 1982) and cholinergic nervous systems (Goyal & Rattan, 1978; Dzieniszewski & Kilbinger, 1978; Gardier *et al.*, 1978; Gallagher *et al.*, 1982).

In airway smooth muscle tissues, neuronal muscarinic receptors attenuate the effects of parasympathetic nerve activity in the guinea-pig lung (Fryer & Maclagan, 1984). In cats, stimulation of the vagus nerves produces bradycardia and bronchoconstriction, as measured from the resulting increase in lung resistance and fall in dynamic lung compliance. Gallamine, a neuromuscular blocking agent which is also known to antagonize the negative inotropic and chronotropic actions of muscarinic agonists in the heart (Laity & Garg, 1962; Muscholl, 1980), potentiated these vagally-mediated changes in lung resistance and dynamic lung compliance. This occurred at doses which block both cardiac muscarinic receptors and neuromuscular transmission (Blaber *et al.*, 1985).

Furthermore, bronchoconstriction induced by intravenously applied acetylcholine was not potentiated by gallamine, thus indicating that post-synaptic muscarinic receptors in the lung may not be involved in the increase in muscle tone induced by gallamine during nerve stimulation. (+)-Tubocurarine or suxamethonium, however, did not affect the response of the lung or the heart to vagal stimulation (Blaber *et al.*, 1985).

The identification and classification of multiple muscarinic receptors in central and peripheral nervous systems has been the object of several investigations using different procedures (for reviews see, Turbanti *et al.*, 1982; Caulfield & Straugham, 1983). Recently, it was reported that pirenzepine, a 'nonclassical' muscarinic antagonist, discriminates between subtypes of muscarinic receptor; that is, in both functional and binding experiments, pirenzepine was found to have a higher affinity for muscarinic receptors of certain brain areas (see reviews by Hammer & Giachetti, 1982; Birdsall & Hulme, 1983) and of sympathetic ganglia (Brown *et al.*, 1980) than for the muscarinic receptors in the isolated ileum or heart (Brown *et al.*, 1980; Fuder *et al.*, 1982; Barlow & Chan, 1982).

To investigate the properties of muscarinic receptors located at the vagus nerve terminals in the airway, we have studied the actions of gallamine and pirenzepine on the pre- and post-junctional muscarinic receptors in dog tracheal muscle tissues. We used these muscarinic antagonists to characterize the muscarinic receptor, since the data obtained are easier to interpret than that when agonists are used (Hammer *et al.*, 1980; Kilbinger *et al.*, 1984).

Methods

Adult mongrel dogs of either sex, weighing 10–15 kg were anaesthetized with intravenous pentobarbitone (10–30 mg kg^{-1}). Segments of the cervical trachea were excised and a strip of transversely running smooth muscle tissue was separated from the cartilage. The mucosa and adventitial areolar tissues were carefully removed. The tracheal smooth muscle was cut in sections, 2.0–2.5 mm wide and about 20 mm long for the double sucrose gap experiments or into strips 15–20 mm long, 1–2 mm wide and 0.3–0.4 mm thick for microelectrode recording. These preparations were bathed in a modified Krebs solution of the following ionic composition (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134.0, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5. The solution was aerated with 97% O_2 and 3% CO_2 and the pH was adjusted to 7.3–7.4. For intracellular recording, a conventional microelectrode filled with 3 M KCl was inserted from the outer surface of the preparation. The chamber in which the muscle preparation was mounted had a volume of 2 ml, and was superfused at a rate of 3 ml min^{-1} at a temperature of 35–36°C.

The double sucrose gap method was used to record both the membrane potential and tension development in the tissue. The chamber used has been described in detail elsewhere (Ito & Tajima, 1981). To produce neurogenic responses, field stimulation was applied by a ring electrode placed in the centre pool of the apparatus. Single and repetitive stimulation was applied, with current pulse of 50–100 μs in duration and about 10–30 V in strength. Drugs were dissolved in Krebs solution and applied to the tissue through the central pool of the double sucrose gap apparatus using a multi-way tap (dead-time approximately 30 s).

To measure the mechanical changes, the tissue was mounted in a 1 ml organ bath through which the test solution, at a temperature of 35°C, flowed continuously. The preparation was placed vertically and the ends were tied with silk thread. One end of the strip was tied to a mechanotransducer (Nihon-Kohden Ltd., RCA-5734) and the other end to a hook at the bottom of the bath. The strips were set up with an initial tension of 0.3 g and mechanical activity was recorded with a pen recorder.

The following drugs were used: gallamine, acetylcholine hydrochloride, indomethacin (Sigma), pirenzepine (Boehringer), propranolol hydrochloride (Nikken Chemical), tetrodotoxin (Sankyo), and atropine sulphate (Daiichi).

Results (amplitude of contractions or e.j.ps) are expressed as mean ± s.d. and were analyzed for statistical significance by Student's *t* test.

Results

Effects of pirenzepine and gallamine on the electrical membrane properties of smooth muscle cells in the dog trachea

The resting membrane potential of tracheal cells was − 60.0 ± 1.5 mV ($n = 50$). Pirenzepine and gallamine each up to 10^{-5} M had no effect on the resting membrane potential (in 10^{-5} M pirenzepine; − 58.6 ± 1.5 mV ($n = 20$) and in 10^{-5} M gallamine; − 59.0 ± 1.5 mV ($n = 25$)).

During application of pirenzepine (10^{-7} M) or gallamine (10^{-5} M), there were no apparent changes in the amplitude of the electrotonic potentials evoked by the constant intensity of inward and outward current pulses applied extracellularly using the microelectrode method. Thus, the input membrane resistance or membrane potential of the smooth muscle cells were unaffected by pirenzepine (10^{-7} M) or gallamine (10^{-5} M).

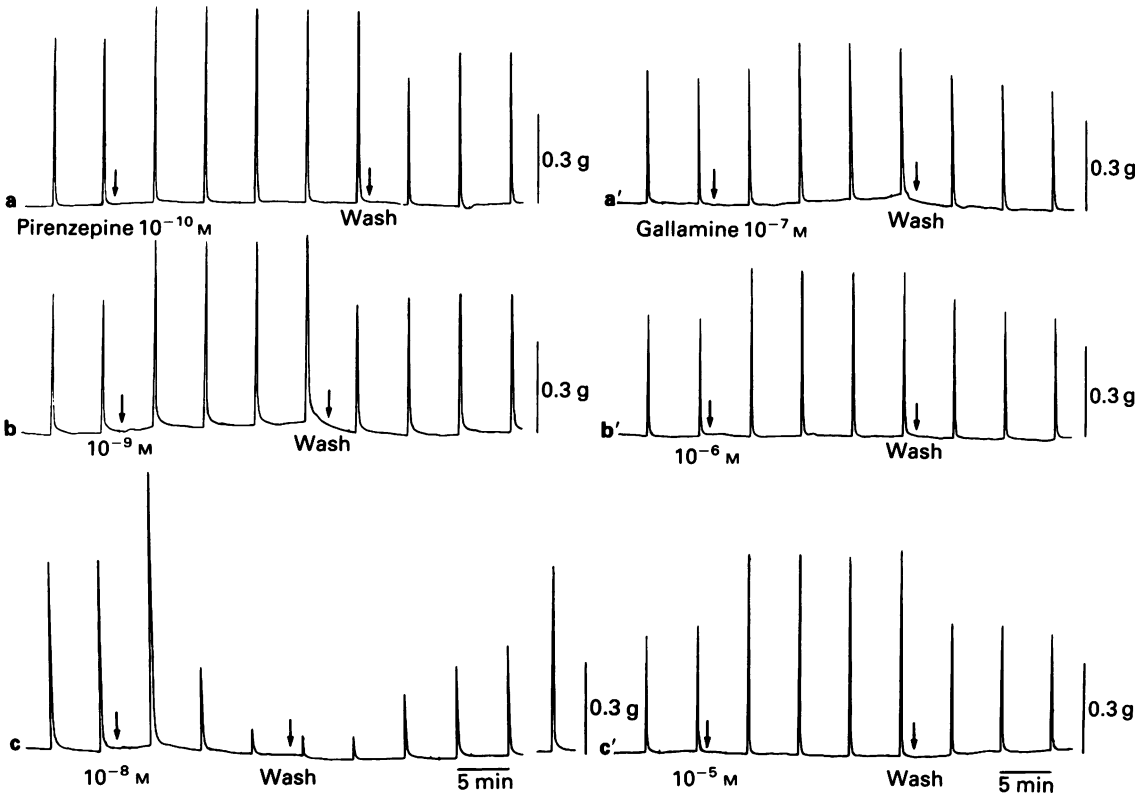

Figure 1 Effects of various concentrations of pirenzepine (10^{-10}–10^{-8} M) and gallamine (10^{-7}–10^{-5} M) on resting tension and twitch contractions evoked by field stimulation (10 stimuli at 20 Hz). Arrows indicate application and removal of the agents. (a–c) Pirenzepine (10^{-10}–10^{-8} M); (a'–c') gallamine (10^{-7}–10^{-5} M).

Effects of pirenzepine and gallamine on twitch contractions evoked by nerve stimulation

To observe the effects of pirenzepine or gallamine on twitch contractions evoked by cholinergic nerve fibres in the dog tracheal tissue, field stimulation of short duration (500 μs) was applied in the combined presence of indomethacin (10^{-5} M) and propranolol (10^{-6} M) (Inoue *et al.*, 1984).

Figure 1 shows the effects of pirenzepine (10^{-10}–10^{-5} M) or gallamine (10^{-7}–10^{-5} M) on the amplitude of twitch contractions evoked by repetitive field stimulation (10 stimuli at 20 Hz) applied every 5 min. Pirenzepine (10^{-10} or 10^{-9} M) slightly increased resting tone and enhanced the amplitude of twitch contractions to 1.14 ± 0.04 ($n = 4$) or to 1.28 ± 0.02 ($n = 6$) times the control, respectively (the mean amplitude of 3 twitch contractions before application of agent was taken as 1.0 and the effects of agents were observed from the third twitch contraction after application of either agent). At a concentration of 10^{-8} M, pirenzepine had dual actions on the twitch contraction evoked by repetitive stimulations. The first twitch contraction evoked after application of pirenzepine was increased to 1.45 ± 0.07 times the control ($n = 5$) and this was followed by a progressive decline (the third twitch contraction; 0.09 ± 0.02 times the control; $n = 5$). Furthermore, at concentrations over 10^{-8} M this agent reduced the resting tension with a reduction in the amplitude of twitch contractions.

Gallamine similarly enhanced the amplitude of twitch contractions, although higher doses (10^{-7}–10^{-5} M) than those of pirenzepine were required. This increase in twitch contractions was dose-dependent and the mean values were 1.17 ± 0.10 times the control at 10^{-7} M ($n = 5$), 1.33 ± 0.08 times at 10^{-6} M ($n = 7$), or 1.62 ± 0.04 times at 10^{-5} M ($n = 5$). During repetitive stimulation in the presence of gallamine (10^{-7} or 10^{-6} M), the resting tone of the muscle tissue slightly increased. At 10^{-5} M, this agent slightly reduced the resting tension but there was no depressant effect on the twitch contraction.

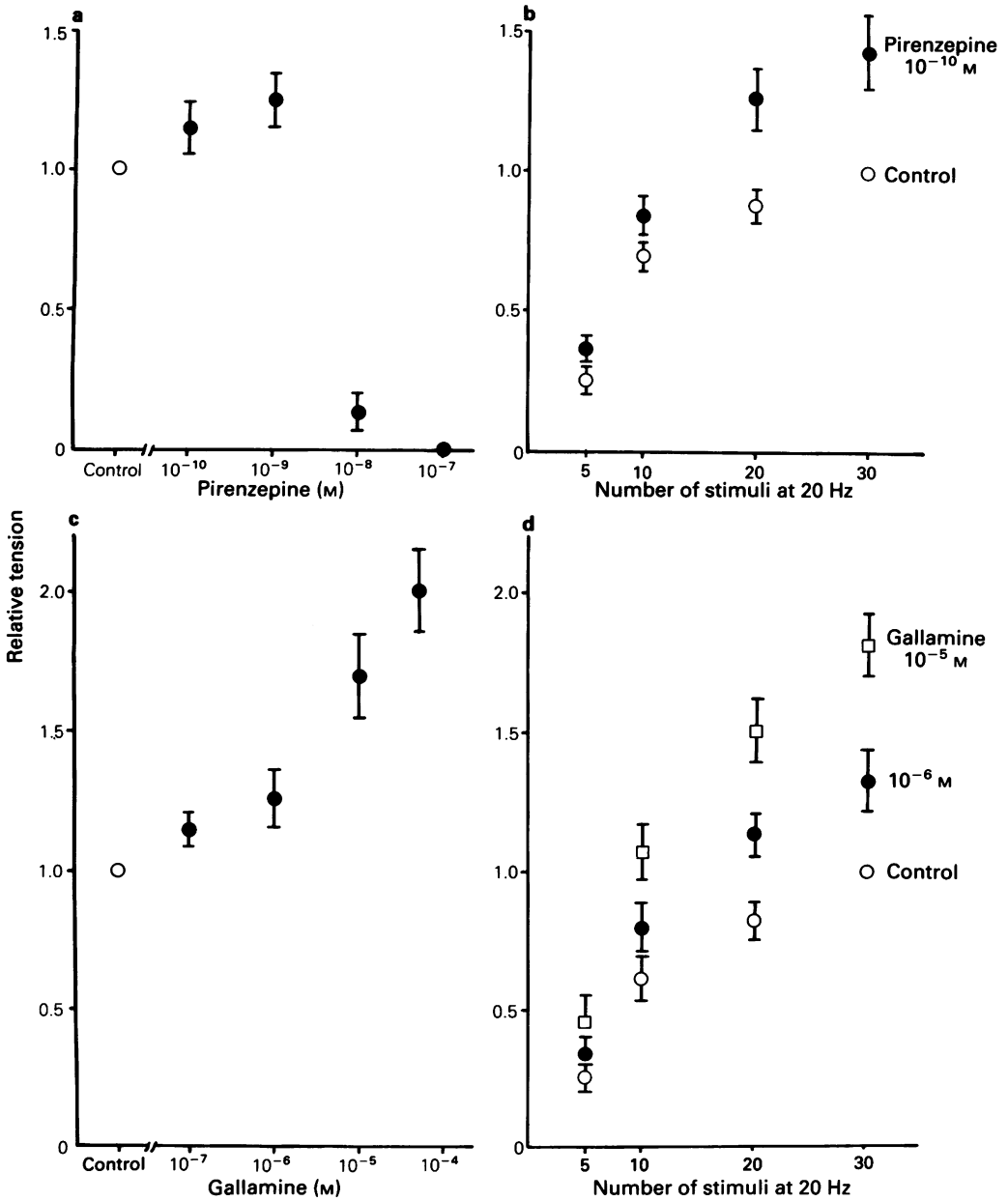

Figure 2 Effects of pirenzepine and gallamine on the relative amplitude of twitch contractions evoked by field stimulations. (a and c) Effects of pirenzepine (a; 10^{-10}–10^{-7} M) and gallamine (c; 10^{-7}–5×10^{-5} M) on the relative amplitude of twitch contractions. The amplitude of twitch contractions evoked by 10 stimuli at 20 Hz in normal Krebs solution was defined as a relative tension of 1.0. (○) Control; (●) various concentrations of pirenzepine or gallamine (b and d) Relationship between the number of stimuli (5–30 at 20 Hz) and relative amplitude of twitch contractions in the presence or absence of pirenzepine (b) and also gallamine (d). The amplitude of twitch contraction evoked by 30 stimuli at 20 Hz in normal Krebs solution was defined as a relative tension of 1.0. (○) Control; (●) pirenzepine 10^{-10} M (b) or gallamine 10^{-6} M; (□) gallamine 10^{-5} M. $n = 4$–6.

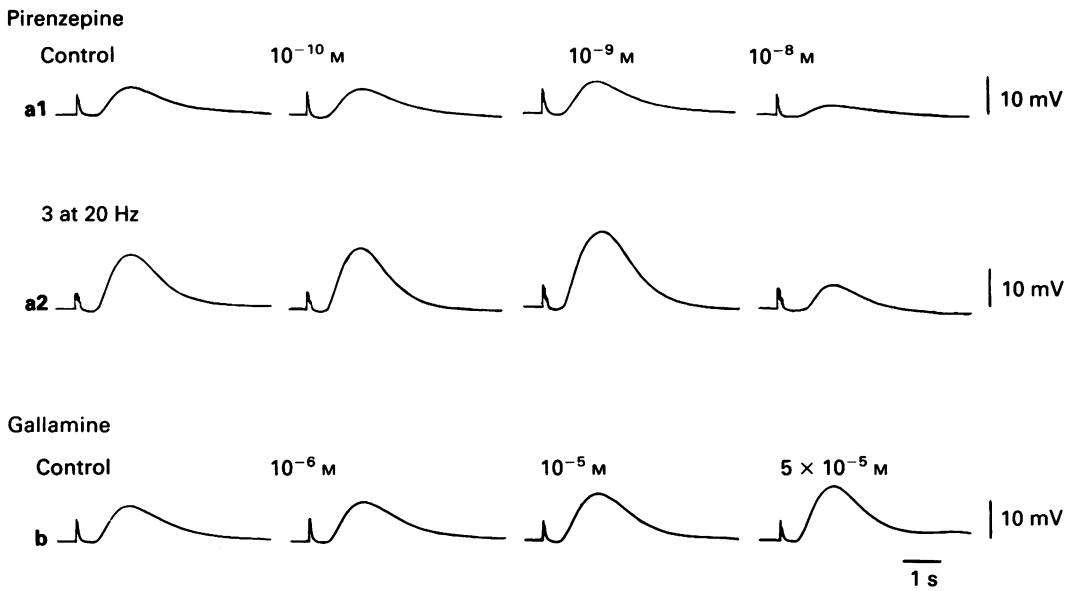

Figure 3 Effects of various concentrations of pirenzepine and gallamine on the amplitude of e.j.ps evoked by single (a_1 and b) or repetitive field stimulation (a_2; 3 pulses at 20 Hz). The pulse duration was 50 μs.

Figures 2a and c show dose-response relationships for the effects of pirenzepine and gallamine on the amplitude of twitch contractions evoked by 10 stimuli at 20 Hz. The evaluation procedures were the same as for Figure 1; i.e. the mean value of the third twitch contraction after application of either agent was measured. Figures 2b and d show the amplitude of the twitch contractions and number of stimuli used to evoke these (20 Hz) in the presence or absence of pirenzepine (10^{-10} M) and gallamine (10^{-6}–10^{-5} M), respectively. The amplitude of twitch contraction evoked by 30 pulses with 20 Hz was normalized as the control. When the number of stimuli was increased, the amplitudes of the contractions increased proportionally. The relationship between twitch contraction amplitude and number of stimuli was shifted to the left.

Effects of pirenzepine and gallamine on the amplitude of the excitatory junction potential (e.j.p.)

To assess the mechanisms involved in the potentiating effects of pirenzepine or gallamine on twitch contractions, the effects of these agents on e.j.ps were examined by the double sucrose gap method in the presence of indomethacin and propranolol (Inoue *et al.*, 1984; Walters *et al.*, 1984).

As shown in Figure 3, field stimulation (50 μs duration) was applied through electrodes placed in the centre pool of the double sucrose gap apparatus and an excitatory junction potential (e.j.p.) was evoked. Pirenzepine (10^{-9} M) slightly, but significantly ($P<0.01$) enhanced e.j.p. amplitude to 1.15 ± 0.07 times the control as measured 5 min after application ($n = 7$). At higher concentrations pirenzepine (10^{-8} M) inhibited the amplitude to 0.20 ± 0.05 times the control ($n = 6$). When repetitive stimuli at a high frequency (3 stimuli at 20 Hz) were applied, potentiation was evident at lower doses of pirenzepine (in 10^{-10} M, the amplitude was increased to 1.13 ± 0.10 times the control, $n = 8$), and at 10^{-9} M, this agent further increased the amplitude of e.j.p. to 1.40 ± 0.18 the control value ($n = 8$). However, at 10^{-8} M, this agent suppressed e.j.p. amplitude to 0.42 ± 0.05 times the control ($n = 6$). Gallamine (10^{-6} and 5×10^{-5} M), progressively increased the amplitude of the e.j.p. evoked by a single stimulus to 1.10 ± 0.05 and 1.40 ± 0.12 times the control, respectively ($n = 6$–8).

Figure 4 summarizes the effects of pirenzepine and gallamine on the relative amplitude of the e.j.p., the input membrane resistance and resting membrane potential of the smooth muscle cells. Low concentration of pirenzepine (10^{-9} M) enhanced and higher concentrations (10^{-8} M) suppressed e.j.p. amplitude with no change in the input membrane resistance and resting membrane potential of the smooth muscle cells. Gallamine (10^{-5} M) significantly enhanced e.j.p. amplitude without changing membrane potential or input membrane resistance (Figure 4b). These electrical effects were accompanied by parallel changes in

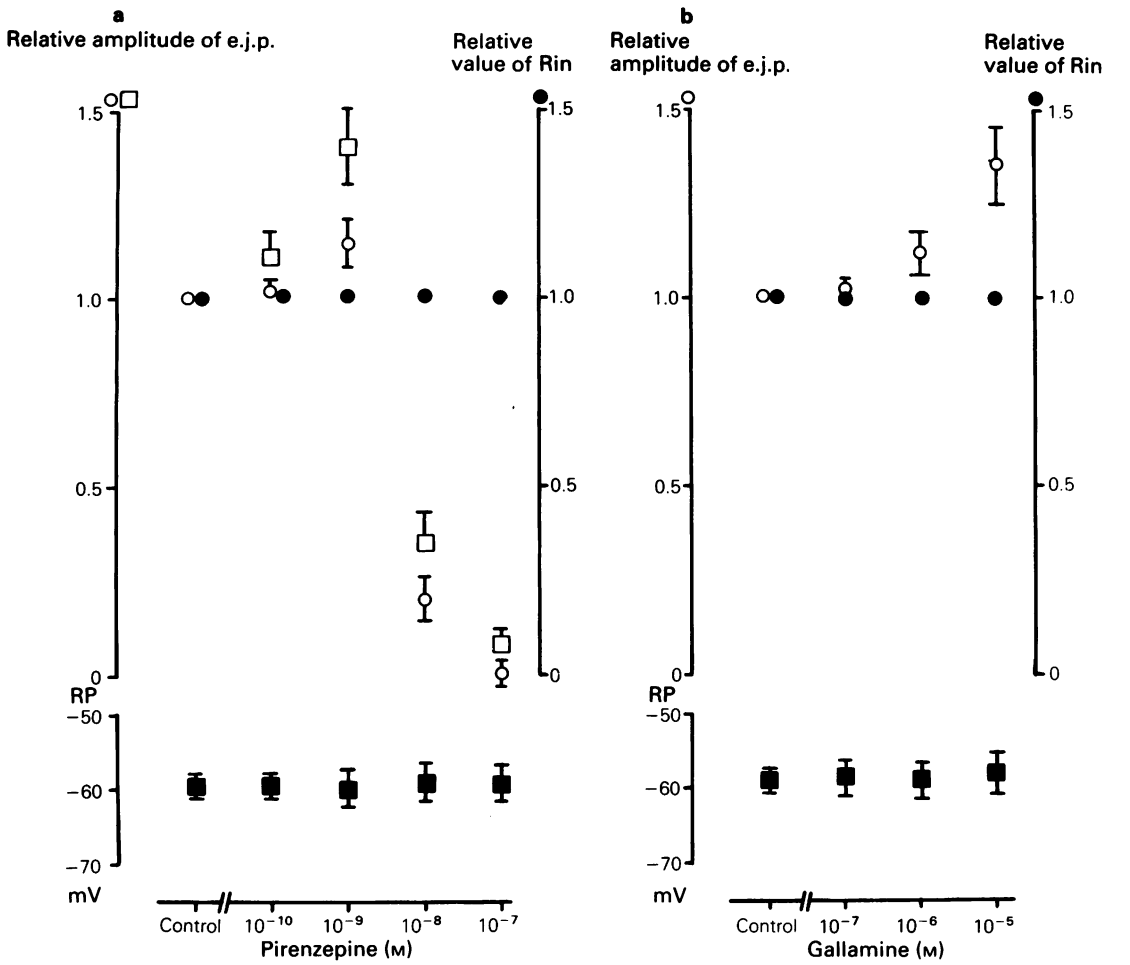

Figure 4 Relationship between the concentration of pirenzepine (a) and gallamine (b) and the relative amplitude of the e.j.p. (○, □), relative value of input membrane resistance (Rin) (●) and the resting membrane potential (RP) (■) of the smooth muscle cells. The amplitude of the e.j.p. evoked by single (○) or repetitive field stimulation in normal Krebs solution (□; 3 pulses at 20 Hz), and the amplitude of the electrotonic potentials produced by square pulses in normal Krebs solution was defined as 1.0. Each point is the mean value derived from 5–20 experiments; vertical bars indicate 2 × s.d.

the twitch contraction evoked by field stimulation.

Effects of pirenzepine and gallamine on the acetylcholine (ACh)-induced contraction of the dog trachea

To clarify the site of actions of pirenzepine or gallamine on this tissue, we examined the effects of both agents on the post-junctional response of the smooth muscle cells to ACh. For this purpose, the tension development induced by application of ACh (10^{-7} M) was measured before and during application of pirenzepine (10^{-10}–10^{-7} M) or gallamine (10^{-6} M–10^{-5} M), together with the twitch contraction evoked by field stimulation.

As shown in Figure 5, the amplitude of the ACh (10^{-7} M)-induced contraction was larger than that of the twitches evoked by 10 stimuli at 20 Hz. Application of gallamine (10^{-6} M) increased the twitch contraction amplitude to 1.25 ± 0.12 times the control ($n = 5$). However, amplitude of the ACh-induced contractions were unaffected. Gallamine (5×10^{-5} M) reduced ACh-induced contractions to 0.80 ± 0.10 times the control ($n = 5$ (Figure 5h)). Pirenzepine

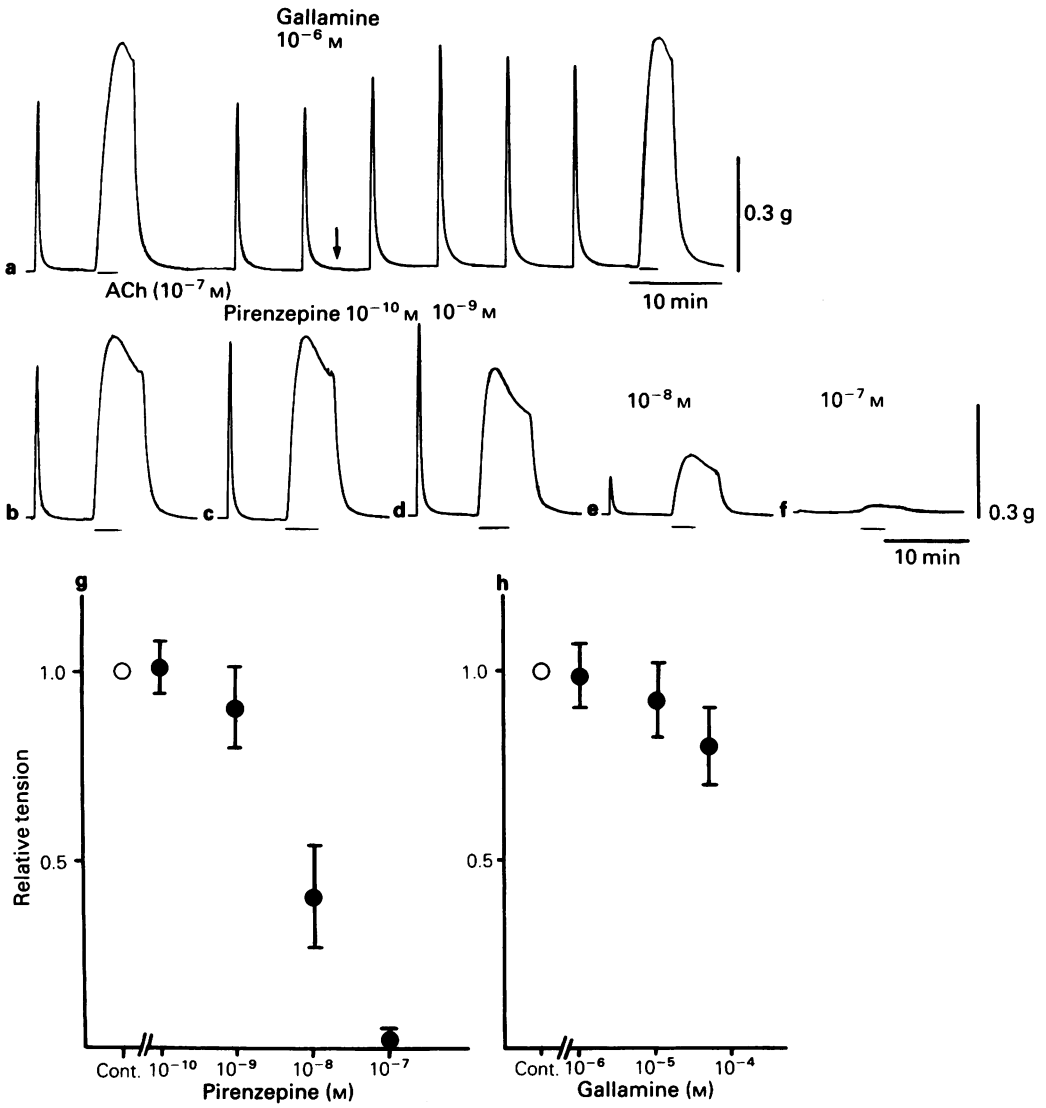

Figure 5 Effects of gallamine (a) and pirenzepine (b–f) on the amplitude of twitch contractions evoked by field stimulation (10 stimuli at 20 Hz) or by acetylcholine (ACh, 10^{-7} M). (a) During repetitive field stimulation (10 stimuli at 20 Hz applied every 5 min), gallamine (10^{-6} M) was applied at the arrow. The amplitude of twitch contractions was enhanced, but the amplitude of ACh-induced contractions was not affected. (b–f) Twitch and ACh-induced contractions in normal Krebs solution (b) and in the presence of various concentrations of pirenzepine (10^{-10}–10^{-7} M) (e–f). Horizontal bars indicate the application of ACh (10^{-7} M). (g and h) Effects of pirenzepine (10^{-10}–10^{-7} M) and gallamine (10^{-6}–5×10^{-5} M) on the relative amplitude of ACh (10^{-7} M)-induced contractions. The amplitude of ACh-induced contractions in normal Krebs solution was defined as a relative amplitude of 1.0.

(10^{-10}–10^{-9} M) enhanced the twitch contraction amplitude evoked, although at concentrations over 10^{-8} M, the twitches were reduced (Figure 5b–f). However, in concentrations over 10^{-9} M, pirenzepine had only a depressant action on amplitude of the ACh-induced contractions (Figure 5g).

These results show that both gallamine (up to 10^{-5} M) and pirenzepine (up to 10^{-9} M) inhibited ACh-induced contraction yet simultaneously enhanced e.j.p. amplitude, indicating both pre- and postsynaptic

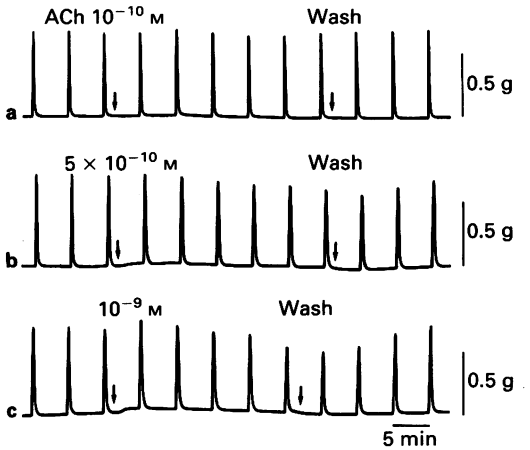

Figure 6 Effects of various concentrations of acetylcholine (ACh) on the twitch contractions evoked by field stimulation (20 Hz, 10 pulses at 5 min intervals). Application and removal of ACh are indicated by arrows. (a) 10^{-10} M, (b) 5×10^{-10} M, (c) 10^{-9} M ACh.

actions of these agents.

Effects of acetylcholine on twitch contractions evoked by nerve stimulation

Pirenzepine (10^{-10}–10^{-9} M) and gallamine (10^{-7} M) each increased e.j.p. and twitch contraction amplitude evoked by field stimulations. It was therefore of interest to observe the effects of ACh on resting tension and twitch contractions. At low concentrations of ACh (10^{-10} M) amplitude of the twitch contractions was slightly reduced whilst at higher concentrations (10^{-9} M) there was an accelerated reduction in twitch contraction amplitude. ACh in concentrations over 5×10^{-10} M, increased the resting tension (Figure 6).

Effects of gallamine on the summation or facilitation process of e.j.ps

Figure 7a shows the effects of gallamine (5×10^{-6} M) on the relationship between the amplitude of e.j.ps and the number of pulses at a stimulus frequency of 20 Hz. The amplitude of e.j.p. evoked by a single stimulation was defined as a relative amplitude of 1.0. When several stimuli were applied, a linear relationship was observed between the number of stimuli and e.j.p. amplitude, with slopes of 1.8 ± 0.3 ($\pm$ s.d., $n = 5$) and 2.2 ± 0.3 ($\pm$ s.d., $n = 4$) in the control and gallamine-treated muscle, respectively. Thus, gallamine enhanced e.j.p. amplitude during repetitive field stimulation.

In contrast e.j.p. amplitude recorded by the double stimulus experiments (5–60 s interval) showed some depression phenomena, i.e. the amplitude of the second e.j.p was smaller than that of the first one, and this depression lasted for more than 1 min (Ito & Tajima, 1981). Gallamine (10^{-5} M) had no effects on this phenomenon (Figure 7b).

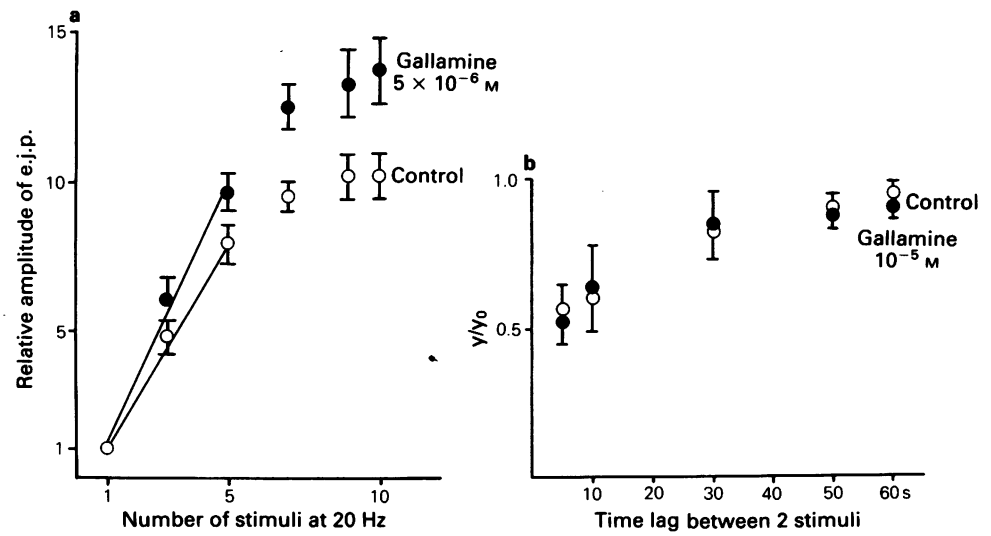

Figure 7 (a) Effects of gallamine on the relationship between e.j.p. amplitude and the number of stimuli at 20 Hz. (○) Control; (●) gallamine (5×10^{-6} M). Each point is the mean value of several experiments, vertical bars indicate $2 \times$ s.d. The straight line was fitted by the method of least squares. (b) Relative changes in the amplitude of the test e.j.p. (the second e.j.p.) following recording of the conditioning e.j.ps (the first e.j.p.) measured at various time intervals in the absence (○) or presence of gallamine 10^{-5} M (●).

Discussion

Blaber *et al.* (1985) reported that pirenzepine and gallamine, both 'nonclassical' muscarinic antagonists, showed no direct action on cat airway smooth muscle cells, yet exerted an indirect action through modulation of ACh release from vagus nerve terminals. In the present study, both pirenzepine and gallamine increased twitch contraction and e.j.p. amplitudes, strongly suggesting that the ACh releasing mechanism at the vagus nerve terminals is under the control of endogenous ACh (an autoregulatory process). If this enhancement of e.j.p. amplitude is due to blockade of a cholinergic autoregulatory process, then it can be assumed that sufficient amounts of endogenous ACh are present in the vicinity of presynaptic muscarinic receptors. In the guinea-pig and bovine trachealis muscle, the anticholinesterase, neostigmine and physostigmine, produce spasm due to an enhancement of the actions of endogenously released ACh and/or with increase in ACh release from the nerve terminals (Carlyle 1963; Kirkpatrick & Rooney, 1982). Therefore in the dog trachealis muscle, it seems probable that small amounts of ACh are spontaneously released during the resting state of the nerve, and that this ACh inhibits the neuronal release of ACh during resting and active states.

The generation of miniature e.j.p. was observed in the dog bronchiole on rare occasions (Inoue & Ito; unpublished observations). On the assumption that there is a basal release of ACh, one might expect a degree of maintained depolarization which could be reversed in the presence of higher concentrations of pirenzepine and gallamine. However, neither drug had any effect on membrane potential although they both apparently caused some reduction in muscle tone. In airway smooth muscle, however, membrane depolarization is not always a key factor in the initiation of contraction especially at low concentration of ACh (Ito & Itoh, 1984). Such concentrations might increase tension through pharmaco-mechanical coupling without a change in membrane potential (Hashimoto *et al.*, 1985).

Similarly, the release of noradrenaline, when measured as inhibitory postsynaptic potentials in the submucous plexus of the guinea-pig, is probably under the control of endogenous ACh, since pirenzepine increased the amplitude of i.p.s.ps (North *et al.*, 1985). There is also evidence that small amounts of ACh are spontaneously released in the form of miniature excitatory postsynaptic potentials in the bull-frog sympathetic ganglia (Tokimasa, 1985).

In the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum, ACh release is also regulated by inhibitory muscarinic receptors on pre-synaptic nerve terminals, since oxotremorine reduces [^{3}H]-ACh outflow evoked by nerve stimulation, in a concentration-dependent manner, and muscarinic antagonists produced parallel shifts of the concentration-response curves of the pre-junctional effects of oxotremorine. However, Kilbinger *et al.* (1984) reported that the absolute amounts of ACh outflow in the presence of muscarinic antagonists were not significantly different from the control values. Therefore, they concluded that autoregulation of ACh release was not of functional importance in the guinea-pig ileum.

Recently, it was demonstrated that muscarinic M_1- and M_2-receptors in the guinea-pig enteric nervous system mediate the depolarization of postsynaptic membranes and presynaptic inhibition of transmitter release, respectively (North *et al.*, 1985). Furthermore, in certain neurones of the rat nucleus parabrachialis, muscarine produced a membrane hyperpolarization, mediated by M_2-receptors (Egan & North, 1986). In contrast, in rat and rabbit hearts there were no differences between the pre- and post-junctional pA_2 values for any of the antagonists tested including pirenzepine (Fuder *et al.*, 1981; 1982). Similarly, in the guinea-pig ileum, it was concluded that although pirenzepine was 30–100 times more potent at M_1- than at M_2-receptors (Hammer *et al.*, 1980; Raiteri *et al.*, 1982; 1984), this agent apparently did not distinguish between pre-junctional neuronal and post-junctional muscular muscarinic receptors, i.e. both types of receptor had the same low affinity for pirenzepine.

In the present experiments, low concentrations of pirenzepine (10^{-9} M) significantly enhanced the amplitude of twitch contractions and e.j.ps recorded from trachealis muscle cells. At higher concentrations (over 10^{-8} M) both these responses were depressed, presumably by suppressing the sensitivity of the muscle membrane to ACh. Gallamine (5×10^{-5} M) also suppressed the sensitivity of the smooth muscle cell to ACh, even though it enhanced the e.j.p. amplitude, thereby indicating that the pre-junctional actions of gallamine may be underestimated on account of its post-junctional effects. Similarly, the pre-junctional effects of pirenzepine (10^{-10}–10^{-9} M) may also be an underestimate. However, it was difficult in the present experiments to distinguish clearly the receptor subtypes in the pre- and post-junctional membranes.

During repetitive field stimulation, e.j.ps showed a depression phenomenon, which could be classified into two components (Ito & Tajima, 1981). The present experiments showed that gallamine enhanced e.j.p. summation, but did not affect the time course of the depression phenomenon (Figures 7a and b), thereby indicating that the depression was not the result of the auto-regulation of ACh release. Presumably, other substances may contribute to this depression.

In dog trachea or bronchioles, endogenous pros-

taglandins also regulate the release of ACh, both in the active and resting states of the nerve terminal (Inoue & Ito, 1984; 1985; Walters *et al.*, 1984). It is also known that low concentrations of endogenous or exogenous catecholamine activate pre- and post-junctional β-adrenoceptors and induce potent bronchodilator effects (Ito & Tajima, 1982). However, in the bronchiolar tissue, histamine acts on pre-junctional terminals of the vagus nerve to enhance the release of ACh through activation of neuronal H_1-receptors (Inoue & Ito, 1986). Thus, neural and humoural factors including ACh itself influence the extent of release of ACh at the airway neuro-effector junction with a resultant decrease or enhancement of cholinergic neurotransmission.

References

BARLOW, R.B. & CHAN, M. (1982). The effects of pH on the affinity of pirenzepine for muscarinic receptors in the guinea-pig ileum and rat fundus strip. *Br. J. Pharmacol.*, **77**, 559–563.

BIRDSALL, N.J.M. & HULME, E.C. (1983). Muscarinic receptor subclasses. *Trend. Pharmacol. Sci.*, **4**, 459–463.

BLABER, L.C., FRYER, A.D. & MACLAGAN, J. (1985). Neuronal muscarinic receptors attenuate vagally-induced contraction of feline bronchial smooth muscle. *Br. J. Pharmacol.*, **86**, 723–728.

BROWN, D.A., FORWARD, A. & MARSH, S. (1980). Antagonist discrimination between ganglionic and ileal muscarinic receptors. *Br. J. Pharmacol.*, **71**, 362–364.

CARLYLE, R.F. (1963). The mode of action of neostigmine and physostigmine on the guinea-pig trachealis muscle. *Br. J. Pharmacol.*, **21**, 137–149.

CAULFIELD, M. & STRAUGHAM, D. (1983). Muscarinic receptors revisited. *Trends Neurosci.*, **6**, 73–75.

DZIENISZEWSKI, P. & KILBINGER, H. (1978). Muscarinic modulation of acetylcholine release evoked by dimethylphenylpiperazinium and high potassium from guinea-pig myenteric plexus. *Eur. J. Pharmacol.*, **50**, 385–391.

EGAN, T.M. & NORTH, R.A. (1986). Acetylcholine hyperpolarises central neurones by acting on an M_2-muscarinic receptor. *Nature*, **319**, 405–407.

FRYER, A.D. & MACLAGAN, J. (1984). Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br. J. Pharmacol.*, **83**, 973–978.

FUDER, H., MEISER, C., WORMSTALL, H. & MUSCHOLL, E. (1981). The effect of several muscarinic antagonists on pre- and post-synaptic receptors in the isolated rabbit heart. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **816**, 31–37.

FUDER, H., RINK, D. & MUSCHOLL, E. (1982). Sympathetic nerve stimulation on the perfused rat heart affinities of n-methylatropine and pirenzepine at pre- and post-synaptic muscarinic receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **318**, 210–219.

GALLAGHER, J.P., GRIFFITH, W.H. & SHINNICK-GALLAGHER, P. (1982). Cholinergic transmission in cat parasympathetic ganglia. *J. Physiol.*, **332**, 473–486.

GARDIER, R.W., TSEVDOS, E.J. & JACKSON, D.B. (1978). The effect of pancuronium and gallamine on muscarinic transmission in the superior cervical ganglion. *J. Pharmacol. Exp. Ther.*, **204**, 46–53.

GOYAL, R.K. & RATTAN, S. (1978). Neurohumoral, hormonal and drug receptors for the lower esophageal sphincter. *Gastroenterology*, **74**, 598–619.

HAMMER, R., BERRIE, E.P., BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature*, **283**, 90–92.

HAMMER, R. & GIACHETTI. (1982). Muscarinic receptor subtypes, M_1 and M_2 biochemical and functional characterization. *Life Sci.*, **31**, 2991–2998.

HASHIMOTO, T., HIRATA, M. & ITO, Y. (1985). A role for inositol 1,4,5-trisphosphate in the initiation of agonist-induced contraction of dog tracheal smooth muscle. *Br. J. Pharmacol.*, **86**, 191–199.

INOUE, T. & ITO, Y. (1985). Pre- and post-junctional actions of prostaglandin I_2 carbocyclic thromboxane A_2 and leukotriene C_4 in dog tracheal tissue. *Br. J. Pharmacol.*, **84**, 289–298.

INOUE, T. & ITO, Y. (1986). Characteristics of neuro-effector transmission in the smooth muscle layer of dog bronchiole and modifications by autacoids. *J. Physiol.*, **370**, 551–565.

INOUE, T., ITO, Y. & TAKEDA, K. (1984). Prostaglandin-induced inhibition of acetylcholine release from neuronal elements of dog tracheal tissue. *J. Physiol.*, **349**, 553–570.

ITO, Y. & TAJIMA, K. (1981). Actions of indomethacin and prostaglandins on neuro-effector transmission in the dog trachea. *J. Physiol.*, **319**, 379–392.

ITO, Y. & TAJIMA, K. (1982). Dual effects of catecholamines on pre- and post-junctional membranes in the dog trachea. *Br. J. Pharmacol.*, **75**, 433–440.

ITO, Y. & ITOH, T. (1984). The roles of stored calcium in contractions of cat tracheal smooth muscle produced by electrical stimulation, acetylcholine and high K^+. *Br. J. Pharmacol.*, **83**, 667–676.

KILBINGER, H., HALIM, S., LAMBRECHT, G., WELLER, W. & WESSLER. (1984). Comparison of affinities of muscarinic antagonists to pre- and post-junctional receptors in guinea-pig ileum. *Eur. J. Pharmacol.*, **103**, 313–320.

KIRKPATRICK, C.T. & ROONEY, R.J. (1982). Contractures produced by carbamate anticholinesterase in bovine tracheal smooth muscle. *Clin. Exp. Pharmacol. Physiol.*, **9**, 603–611.

LAITY, J.H.L. & GARG, B.K. (1962). A note on the action of gallamine on isolated rabbit auricles. *J. Pharm. Pharmacol.*, **14**, 371–373.

MUSCHOLL, E. (1980). Peripheral muscarinic control of norepinephrine release in the cardiovascular system. *Am. J. Physiol.*, **239**, H713–H720.

NORTH, R.A., SLACK, B.E. & SURPRENANT, A. (1985). Muscarinic M_1 and M_2 receptors mediate depolarization and presynaptic inhibition in guinea-pig enteric nervous system. *J. Physiol.*, **368**, 435–452.

RAITERI, M., MARCHI, M. & MAURA, G. (1982). Presynaptic muscarinic receptors increase tritiated dopamine release

evoked by "quasi physiological" depolarization. *Eur. J. Pharmacol.*, **83,** 127–129.

RAITERI, M., LEARDI, R. & MARCHI, M. (1984). Heterogeneity of presynaptic muscarinic receptors regulating neurotransmitter release in the rat brain. *J. Pharmacol. Exp. Ther.*, **228,** 209–214.

STARKE, K. (1977). Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmacol.*, **77,** 1–124.

TOKIMASA, T. (1985). Spontaneous muscarinic suppression of the Ca-activated K-current in bullfrog sympathetic neurones. *Brain Research.*, **344,** 134–141.

TURBANTI, L., SUBISSI, A. & BRAMANTI, G. (1982). Antimuscarinic drugs; Traditional and potential new-therapeutic agents. *Pharmacol. Res. Commun.*, **14,** 923–940.

WALTERS, E.H., OBYRNE, P.M., FABBI, L.M., GRAF, P.D., HOLTZMAN, M.J. & NADEL, J.A. (1984). Control of neurotransmission by prostaglandins in canine trachealis smooth muscle. *J. Appl. Physiol. Resp. Environ. Exc. Physiol.*, **57,** 129–134.

WATSON, M., YAMAMURA, A.I. & ROESKE, W.R. (1983). A unique regulatory profile and regional distribution of ^{3}H-pirenzepine binding in the rat provide evidence for distinct M_1 and M_2 muscarinic subtypes. *Life Sci.*, **31,** 2019–2023.

(Received May 27, 1987.
Revised September 3, 1987.
Accepted October 12, 1987.)