Identification of M₁ muscarinic receptors in pulmonary sympathetic nerves in the guinea-pig by use of pirenzepine

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1 The effect of pirenzepine, a muscarinic antagonist considered to be selective for M₁ receptors, was studied on bronchoconstriction and bradycardia elicited by preganglionic stimulation of the parasympathetic vagal nerves and by i.v. injections of acetylcholine (ACh) in anaesthetized guinea-pigs.

2 Pirenzepine was equipotent in the heart and lung as an antagonist of the effects of i.v. ACh at postjunctional muscarinic receptors. Doses of pirenzepine in excess of 1 $\mu\text{mol kg}^{-1}$ abolished all muscarinic responses consistent with non-selective blockade of M₃ receptors on airway smooth muscle and M₂ receptors on atrial cells.

3 In the lung, low doses of pirenzepine (1–100 nmol kg^{-1}) increased vagally-induced bronchoconstriction despite concurrent partial blockade of the postjunctional receptors. This suggests blockade of neuronal muscarinic receptors.

4 Propranolol (1 mg kg^{-1}) increased control bronchoconstrictor responses elicited by ACh and vagal stimulation but did not alter the potency of pirenzepine for postjunctional receptors in heart or lung. However, pirenzepine-induced enhancement of vagally-induced bronchoconstriction was abolished by propranolol, suggesting that pirenzepine may be an antagonist for muscarinic receptors located in the sympathetic nerves innervating airway smooth muscle.

5 These results confirm that bronchoconstrictor stimuli indirectly initiate activation of an opposing sympathetic reflex in the guinea-pig lung. This response is facilitated by muscarinic receptors located in the sympathetic nervous pathway.

6 The high potency of pirenzepine for the neuronal receptors in the sympathetic nerves suggests that these are M₁ receptors. In contrast, the parasympathetic nerves innervating airway smooth muscle in this species contain M₂ receptors which inhibit neurotransmission.

Introduction

In the lung, the dominant nervous control of airway diameter is exerted by parasympathetic nerves which release acetylcholine (ACh) onto muscarinic receptors on the airway smooth muscle cell. Muscarinic receptors also exist on the post-ganglionic terminals of these cholinergic nerves. These neuronal receptors inhibit transmitter release (Fryer & MacLagan, 1984; 1987a; Faulkner *et al.*, 1986) and consequently their blockade with muscarinic antagonists increases vagally-induced bronchoconstriction. This may be a potential hazard when muscarinic antagonists such

as ipratropium are used clinically as bronchodilators (Fryer & MacLagan, 1987b). As muscarinic antagonists affect both prejunctional and postjunctional muscarinic receptors, the degree of blockade of vagally-induced bronchoconstriction produced by these drugs is dependent on the balance between their pre- and postjunctional blocking activity. Thus, it is important to establish whether the neuronal muscarinic receptors belong to the same or to a different receptor subtype from the receptors on the smooth muscle cell.

Muscarinic receptors have been classified into three types on the basis of ligand binding and functional studies by use of selective antagonists. M₁ receptors exhibit a high affinity for pirenzepine and are located mainly in neural tissue (Hammer, 1982),

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whereas the muscarinic receptors located on nerves, glands, heart and smooth muscle exhibit a low affinity for pirenzepine. These have been further subdivided by the use of gallamine which is selective for the M_2 receptors in the heart (Riker & Wescoe, 1951) and by dicyclomine and 4-diphenylacetoxy-N-methyl piperidine (4-DAMP) which displays high affinity for the M_3 receptors on ileal smooth muscle and glands (de Jonge *et al.*, 1986; Birdsall & Hulme, 1984). The latter subtype includes the postjunctional receptors on airway smooth muscle (Barlow *et al.*, 1972).

The high potency of gallamine for the pulmonary neuronal receptors in the guinea-pig and cat (Fryer & MacLagan, 1987a; Blaber *et al.*, 1985) suggests that these receptors may resemble the cardiac M_2 muscarinic receptors. However, the effect of pirenzepine has not been tested in the pulmonary autonomic nerves in these species. The present experiments were carried out to examine the effect of pirenzepine in the guinea-pig in an attempt to clarify the subtype of muscarinic receptor found in the pulmonary autonomic nerves.

Methods

Guinea-pigs (250–300 g, either sex) of the Dunkin Hartley strain were anaesthetized with urethane (1.5 g kg^{-1} , i.p.). The right carotid artery was cannulated for the measurement of blood pressure and heart rate was derived electronically from the blood pressure signal. A cannula was placed in the left jugular vein for the injection of drugs. Both vagi were cut and the distal ends placed on shielded platinum electrodes immersed in a pool of liquid paraffin. Each animal's body temperature was maintained at 37°C by use of a CFP 8185 homeothermic blanket control.

The anaesthetized animals were paralysed with suxamethonium chloride ($100 \mu\text{g kg}^{-1}$) injected every 10 min and respired with positive pressure, constant volume ventilation from a Harvard Rodent respirator. Airflow ($\dot{V}$) and tidal volume (V_t) were recorded as described by MacLagan & Ney (1979). Pulmonary inflation pressure (Ppi) was measured with a Statham pressure transducer (PM5E): one port of the transducer was connected to a side arm of the tracheal cannula and the other port was open to the atmosphere. All signals were displayed on a Gould Recorder and arterial blood samples were taken at regular intervals and analysed for O_2 and CO_2 tension and pH with a Corning 166 microsample blood gas analyser. The tracheal cannula used had a resistance of $40\text{--}60 \text{ cmH}_2\text{O} \cdot 0.1 \text{ s}^{-1}$, so a positive

pressure of approximately $80\text{--}100 \text{ mmH}_2\text{O}$ was needed at each stroke of the pump for adequate ventilation of the animal.

Bronchoconstriction elicited by i.v. ACh or by vagal nerve stimulation was recorded as described by Fryer & MacLagan (1984) as an increase in Ppi over the basal insufflation pressure change produced by the pump. The sensitivity of the method was increased by using a computerized device to subtract the basal tracheal insufflation pressure. The 'subtractor' was re-set before each bronchoconstrictor stimulus and the increase in Ppi which occurred during the ensuing bronchoconstriction was recorded on a second channel of the Gould recorder at higher amplification. The changes in pressure in the trachea recorded in this way probably reflect changes in both resistance and compliance components of the lungs.

Bronchoconstriction and bradycardia were elicited either by intravenous injection of ACh, or by preganglionic stimulation of both cervical vagus nerves (30 Hz, 0.2 ms, 5 s $10\text{--}20 \text{ V}$). Pirenzepine was injected i.v. in cumulative doses. Following each dose of pirenzepine, the responses to nerve stimulation and ACh were measured alternately at 2 min intervals.

All results were expressed as the ratio of the response to agonist or nerve stimulation after the antagonist (B2) divided by the response before administration of the antagonist (B1).

The experiments were repeated in a second group of animals which received propranolol (1 mg kg^{-1} , i.v.) 30 min before administration of cumulative doses of pirenzepine.

Statistical procedures

Results are expressed as mean values $\pm$ s.e. mean from n experiments. Differences between means were tested for significance by use of the unpaired t test and values of $P < 0.05$ were taken as significant.

Drugs

Urethane and suxamethonium chloride (Sigma), acetylcholine bromide (BDH), pirenzepine hydrochloride (Boehringer) and propranolol hydrochloride (ICI) were used. All drugs were dissolved in 0.9% NaCl solution (saline).

Results

The effect of pirenzepine was studied on bronchoconstriction and bradycardia elicited either by intra-

venous injection of ACh, or by stimulation of both cervical vagus nerves. The responses induced by i.v. ACh were used as a measure of postjunctional muscarinic receptor activation in the lungs and heart.

Dose-response curves for bronchoconstriction induced by vagal nerve stimulation were obtained at the start of each experiment and stimulus parameters were then selected for the rest of the experiment (control response, see Figure 1) which produced approximately 50% of the maximal response. A dose of ACh was then selected to cause an increase in Ppi comparable to that produced by the control stimulus

to the vagus nerve. In separate experiments saline injections were used instead of pirenzepine and the results showed that repeated alternating application of ACh or vagal nerve stimulation elicited reproducible bronchoconstrictor responses for several hours (Fryer & MacLagan, 1987a).

Administration of pirenzepine in cumulative doses reduced the bronchoconstriction induced by i.v. ACh, as shown by a decrease in the B_2/B_1 ratio, due to blockade of the muscarinic receptors located post-junctionally on the heart and on airway smooth muscle (Figure 1). In each experiment, the dose of pirenzepine that produced 50% inhibition of the

Figure 1 Summary of results showing the effect of pirenzepine on bronchoconstriction (measured as an increase in pulmonary inflation pressure (Ppi) and bradycardia induced by vagal nerve stimulation (VS; 30 Hz, 0.2 ms, 3 s, 10–20 V; $\blacktriangle$) or by intravenous acetylcholine (ACh $1 \mu\text{g kg}^{-1}$; $\bullet$). The control responses (B₁) to VS (hatched columns) and ACh (open columns) before pirenzepine are shown on the left. Results are expressed in the graphs as the ratio B_2/B_1 where B₂ is the response after pirenzepine.

A second group of guinea-pigs was pretreated with propranolol (1 mg kg^{-1} , open symbols), which abolished the pirenzepine-induced potentiation of vagally-induced bronchoconstriction. The effect of pirenzepine in the heart was not significantly altered by propranolol (not shown but see Table 1). Note that the control bronchoconstrictor responses were potentiated by propranolol. (Mean results with s.e.mean shown by vertical bars; $n = 6$).

Table 1 Antagonist activity of pirenzepine in lung and heart

Response	Pirenzepine ED ₅₀ (nmol kg ⁻¹)	
<i>Bronchoconstriction</i>	<i>Control</i>	<i>After propranolol</i>
ACh-induced	69 ± 30	27 ± 13
Vagally-induced	382 ± 117	63 ± 20**
<i>Bradycardia</i>		
ACh-induced	116 ± 56	152 ± 70
Vagally-induced	491 ± 249	847 ± 429

Dose of pirenzepine causing 50% inhibition of responses induced by acetylcholine (ACh) and by vagal stimulation in heart and lung (ED₅₀, nmol kg⁻¹). Values were calculated for each individual guinea-pig and the table shows the mean ED₅₀ ± s.e.mean (*n* = 6) for a control group of animals compared with a second group that received propranolol (1 mg kg⁻¹) at the start of the experiment. Baseline values for the two groups, in absolute units, are given in Figure 1.

** Different from control: *P* < 0.002.

responses was calculated (ED₅₀) and the mean ED₅₀ values for 5 experiments are summarized in Table 1. These results show that pirenzepine was approximately equipotent as an antagonist for the postjunctional muscarinic receptors located on airway smooth muscle and on the atrial cell.

Effect of pirenzepine on vagally-induced bronchoconstriction and bradycardia

In the same experiments, the effects of pirenzepine were tested on vagally-mediated responses (Figure 1). In the heart, vagally-induced bradycardia was progressively inhibited by increasing doses of pirenzepine in parallel with the inhibition of ACh-induced bradycardia. This is consistent with blockade of the postjunctional muscarinic receptors on the atrial cell.

However, in the lung vagally-induced bronchoconstriction was increased by low doses of pirenzepine, occurring at doses below 100 nmol kg⁻¹, when the postjunctional receptors were either unaffected or partially blocked (Figure 1). As the dose of pirenzepine increased above 100 nmol kg⁻¹, vagally-induced bronchoconstriction gradually decreased and was finally abolished concurrently with inhibition of the response to ACh due to blockade of the postjunctional receptors.

One consequence of this enhancement of vagally-mediated bronchoconstriction by pirenzepine was that the ED₅₀ for pirenzepine as an antagonist of

vagally-induced bronchoconstriction was higher than that needed to inhibit ACh-induced bronchoconstriction (Table 1).

In a second group of animals, the effect of pirenzepine was studied after pretreatment with propranolol (1 mg kg⁻¹, i.v.). The control bronchoconstrictor responses elicited by both ACh and vagal stimulation were increased by propranolol (see control responses in Figure 1). Despite this change, the potency of pirenzepine for the postjunctional receptors in the lung was unaltered by β -adrenoceptor blockade. There was no significant change in the potency of pirenzepine for vagally-mediated or ACh-induced responses in the heart (Table 1). However, pirenzepine no longer caused an increase in vagally-induced bronchoconstriction after propranolol pretreatment (Figure 1, Table 1).

Discussion

The results presented in this paper based on functional tests in the guinea-pig demonstrate that postjunctional muscarinic receptors in the heart and lung have similar affinity for pirenzepine *in vivo*. This is in agreement with previous work both *in vivo* and *in vitro* showing that pirenzepine can be used as a selective antagonist for M₁ receptors but does not distinguish between M₂ and M₃ muscarinic receptors (see Eglen & Whiting, 1986).

It is known from previous work that muscarinic receptors also exist on the parasympathetic nerve terminals innervating airway smooth muscle in several species including guinea-pig (Fryer & MacLagan, 1984), cat (Blaber *et al.*, 1985), dog (Ito & Yoshitomi, 1988) and man (Minette & Barnes, 1987). These muscarinic autoreceptors exert an inhibitory effect on pulmonary parasympathetic neurotransmission so that blockade with an antagonist potentiates vagally-mediated bronchoconstriction. Thus, as the effect of muscarinic antagonists on vagally-induced bronchoconstriction depends on the balance between their prejunctional and postjunctional antagonist action, the potency for the prejunctional receptors will be underestimated (Fryer & MacLagan, 1987a).

Gallamine is a potent antagonist for the pulmonary neuronal receptors in all of the above species suggesting that these receptors probably resemble the cardiac muscarinic receptors and differ from the receptors on airway smooth muscle where the potency of gallamine is low. Thus the neuronal receptors in pulmonary nerves appear to be similar to the muscarinic receptors mediating prejunctional inhibition in other autonomic nerves which are

known to be of the cardiac M_2 subtype (Ashe & Yarosh, 1984; Newberry *et al.*, 1985; Egan & North, 1986).

In view of these findings, it was surprising that the selective M_1 antagonist, pirenzepine, increased vagally-induced bronchoconstriction at low doses as described in this paper. In order to block the post-junctional M_3 muscarinic receptors on airway smooth muscle and the M_2 receptors on the atrial cell, the dose of pirenzepine had to be increased ten fold. This suggests that the pirenzepine-induced enhancement of bronchoconstriction due to vagal stimulation may be due to blockade of M_1 receptors, with a high affinity for pirenzepine, located in the pulmonary autonomic nerves.

The results with pirenzepine differed in one important respect from those previously obtained with gallamine in this species. Propranolol abolished the pirenzepine-induced enhancement of vagal stimulation, whereas such an effect of gallamine was unaltered by β -adrenoceptor blockade (Fryer & MacLagan, 1984). This suggests that these pirenzepine-sensitive M_1 receptors must be located in the sympathetic nervous pathway (see Figure 2), in contrast to the gallamine-sensitive receptors, which are located on the parasympathetic nerve terminals innervating airway smooth muscle (Faulkner *et al.*, 1986).

Stimulation of pulmonary sympathetic nerves causes bronchodilatation *via* activation of β -adrenoceptors on airway smooth muscle; there is also evidence that the sympathetic nerves may inhibit parasympathetic neurotransmission at the ganglia (Partanen *et al.*, 1982) and at the prejunctional cholinergic nerve terminals (Vermeire & Vanhoutte, 1979). Blockade by pirenzepine of M_1 receptors which facilitate neurotransmission in the sympathetic nerves would, therefore, reduce nor-adrenaline release and indirectly increase the effect of parasympathetic nerve stimulation. These M_1 receptors could be located either on the final terminals of the sympathetic postganglionic neurones or at the ganglia (see Figure 2). In the latter case, they would be comparable with the M_1 receptors with a facilitatory role that have been described in sympathetic ganglia in several species (Brown *et al.*, 1980; Mutschler & Lambrecht, 1984; Ashe & Yarosh, 1984; Newberry *et al.*, 1985). Activation of these receptors probably closes a specific subset of voltage-sensitive K^+ channels (Brown, 1983). It is interesting that this facilitatory mechanism is absent from parasympathetic ganglia (Brown, 1988).

These results also imply that the sympathetic nerves must have been activated whenever bronchoconstriction was elicited by vagal nerve stimulation or by injected ACh. This conclusion is supported by the finding described in this paper that the control

Figure 2 Proposed location of muscarinic receptors in autonomic nerves innervating heart and lung. M_1 -pirenzepine-sensitive facilitory receptors: M_2 -gallamine-sensitive inhibitory receptors: M_3 receptors on airway smooth muscle: β -adrenoceptors on cholinergic nerves and airway smooth muscle. The ganglionic nicotinic receptors have been omitted.

bronchoconstrictor responses to ACh and to vagal nerve stimulation were increased after propranolol pretreatment due to removal of the bronchodilator influence of the sympathetic nervous system.

Evidence for reflex activation of the pulmonary sympathetic nerves has been reported previously in the guinea-pig by McCulloch *et al.* (1967) using histamine as the bronchoconstrictor agent. It is not

clear whether this reflex is triggered by afferent nerve stimulation or by anoxia *per se* but the afferent limb of the reflex pathway must lie outside the vagal nerves as, in the experiments described in the present paper and in those of McCulloch *et al.* (1967), the animals were vagotomized.

The distribution of M_1 muscarinic receptors in the autonomic nerves supplying the lung appears to vary between different species. Although it was not possible to demonstrate M_1 receptors in the parasympathetic nerves innervating airway smooth muscle in the guinea-pig, such receptors have been demonstrated in these nerves in the dog (Ito & Yoshitomi, 1988). It has been suggested that M_1 receptors exist in the rabbit pulmonary parasympathetic nerves because pirenzepine was apparently a potent antagonist of vagally-induced bronchoconstriction in this species (Bloom *et al.*, 1987). However, the interpretation of the results in the rabbit is complicated by the fact that the influence of the sympathetic nervous system and blockade of postjunctional receptors were not directly excluded in the latter experiments. M_2 receptors, on the other hand, appear to exist in pulmonary parasympathetic nerves in many species. They have been demonstrated in parasympathetic nerves in the guinea-pig (Fryer & MacLagan, 1984), cat (Blaber *et al.*, 1985), dog (Ito & Yoshitomi, 1988) and human (Minette & Barnes, 1987). In each of these examples, the receptors exerted an inhibitory control on cholinergic neurotransmission to airway smooth muscle. This autoreceptor mechanism appears to be lacking in the rabbit (MacLagan & Faulkner, 1989) and in asthmatic patients (Minette *et al.*, 1988).

Figure 2 shows the mechanisms that underlie the results presented in this paper and in previous work from this laboratory showing that M_1 muscarinic

receptors with a facilitatory function exist in the sympathetic nervous pathway in the guinea-pig lung (this paper) and M_2 receptors exist in the parasympathetic nerves in this species (Fryer & MacLagan, 1984). These M_2 receptors resemble the cardiac M_2 receptors as shown by the antagonist potency of gallamine (Fryer & MacLagan, 1987a).

As the sympathetic nervous system exerts an inhibitory effect on parasympathetic neurotransmission in the guinea-pig, any procedure that reduces the influence of the sympathetic nerves would be expected to increase the bronchoconstrictor effect of the cholinergic pathway. This explains why both M_1 antagonists and β -adrenoceptor blocking drugs (MacLagan & Ney, 1979) potentiate vagally-induced bronchoconstriction in this species.

In man, the existence of M_1 receptors in the sympathetic pathway to the lung has not been investigated but it is known that prejunctional β -adrenoceptors exist in human pulmonary parasympathetic nerves (Rhoden *et al.*, 1988). If the sympathetic nervous system exerts an inhibitory effect on parasympathetic neurotransmission in man, comparable to that shown in the guinea-pig, this could provide an explanation for the bronchospasm provoked by β -adrenoceptor blocking drugs in asthmatic patients, but not in normal subjects. Blockade of the prejunctional β -adrenoceptors should cause an increase in ACh release. In normal individuals this would activate the muscarinic autoreceptors to limit transmitter output to the airway smooth muscle. In asthmatic patients however, where there is an apparent absence of prejunctional M_2 receptors, β -adrenoceptor blockade might result in an increase in ACh release which would exacerbate bronchoconstriction.

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