Pharmacological characterization of the muscarinic receptor antagonist, glycopyrrolate, in human and guinea-pig airways

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1 In this study we have evaluated the pharmacological profile of the muscarinic antagonist glycopyrrolate in guinea-pig and human airways in comparison with the commonly used antagonist ipratropium bromide.

2 Glycopyrrolate and ipratropium bromide inhibited EFS-induced contraction of guinea-pig trachea and human airways in a concentration-dependent manner. Glycopyrrolate was more potent than ipratropium bromide.

3 The onset of action (time to attainment of 50% of maximum response) of glycopyrrolate was similar to that obtained with ipratropium bromide in both preparations. In guinea-pig trachea, the offset of action (time taken for response to return to 50% recovery after wash out of the test antagonist) for glycopyrrolate ($t_{1/2}$ [offset] = 26.4 ± 0.5 min) was less than that obtained with ipratropium bromide (81.2 ± 3.7 min). In human airways, however, the duration of action of glycopyrrolate ($t_{1/2}$ [offset] > 96 min) was significantly more prolonged compared to ipratropium bromide ($t_{1/2}$ [offset] = 59.2 ± 17.8 min).

4 In competition studies, glycopyrrolate and ipratropium bromide bind human peripheral lung and human airway smooth muscle (HASM) muscarinic receptors with affinities in the nanomolar range (K_i values 0.5–3.6 nM). Similar to ipratropium bromide, glycopyrrolate showed no selectivity in its binding to the M_1–M_3 receptors. Kinetics studies, however, showed that glycopyrrolate dissociates slowly from HASM muscarinic receptors (60% protection against [^{3}H]-NMS binding at 30 nM) compared to ipratropium bromide.

5 These results suggest that glycopyrrolate bind human and guinea-pig airway muscarinic receptors with high affinity. Furthermore, we suggest that the slow dissociation profile of glycopyrrolate might be the underlying mechanism by which this drug accomplishes its long duration of action.

Keywords: Human peripheral lung; human airway smooth muscle; glycopyrrolate; ipratropium bromide; muscarinic receptor antagonists; guinea-pig trachea

Abbreviations: [^{3}H]-NMS, [N-methyl-^{3}H]-scopolamine; COPD, chronic obstructive pulmonary disease; EFS, electrical field stimulation; HASM, human airway smooth muscle

Introduction

In mammalian airways, the dominant neural bronchoconstrictor mechanism is provided by the cholinergic nerves (Barnes, 1986). Of the five cloned muscarinic receptor subtypes, mammalian airways express four receptor subtypes (M_1, M_2, M_3 and M_4) that are differentially distributed in the airways (Lazareno et al., 1990; Haddad et al., 1991; 1994a,b; 1996; Mak et al., 1992; Ramnarine et al., 1996). M_1-receptors are found in parasympathetic ganglia where they facilitate neurotransmission and to alveolar walls where their function remains unclear. M_2-receptors are localized to the post-ganglionic cholinergic nerve terminals and provide a functional negative feedback modulation of acetylcholine release (auto-receptors) (Patel et al., 1995). Muscarinic M_2 receptors are also found in high proportion in airway and non-airway smooth muscle where they may be involved in functional antagonism, although this does not seem to be the case in human airways (Eglen et al., 1996; Zaagsma et al., 1997). Excitatory M_3-receptors localized to airway smooth muscle and mucosal

glands mediate bronchoconstriction and mucus secretion respectively (Barnes et al., 1995; Haddad & Rousell, 1998). In rabbit lung, the occurrence of M_4-receptors has been demonstrated but its role in airway function remains to be elucidated (Lazareno et al., 1990; Mak et al., 1993).

Cholinergic neural tone is the major reversible component in the bronchoconstiction observed in chronic obstructive pulmonary disease (COPD) (Gross & Skorodin, 1984a,b; Gross, 1988) and, through the circadian variation of vagal tone, also contributes to nocturnal asthma (Coe & Barnes, 1986; Morrison et al., 1988). Anticholinergics are not very effective in the treatment of chronic asthma, but are more effective in the treatment of asthma exacerbations, suggesting that cholinergic mechanisms may become more important during acute exacerbations (O'Driscoll et al., 1989). The duration of action of the present anticholinergic drugs is sometimes insufficient to provide convenient maintenance therapy for patients or to control nocturnal asthma. Therefore, there is a clinical requirement to develop new drugs of this class.

Glycopyrrolate, a quaternary ammonium compound, is a muscarinic receptor antagonist which has been used in pre-anaesthetic medication to reduce gastric acid and salivary

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secretions or to reverse neuromuscular blockade (Norgaard *et al.*, 1970; Mirakhur & Dundee, 1983). Recently, glycopyrrolate has been used as a bronchodilator in patients with asthma and COPD (Walker *et al.*, 1987; Gilman *et al.*, 1990; Tzelepis *et al.*, 1996). In this study we have investigated the potency and duration of action of glycopyrrolate in inhibiting cholinergic contractile responses evoked by electrical field stimulation (EFS) in guinea-pig, and human airways. Parallel studies have investigated the antagonist affinity and the time taken for dissociation of the drug from muscarinic receptors in order to evaluate its possible clinical role as a bronchodilator for the treatment of reversible airways obstruction in patients with COPD and in acute exacerbations of asthma. The pharmacological profile of glycopyrrolate was also determined in rat cerebral cortex using [^{3}H]-telenzepine to label M_1-receptors and in rat heart and salivary glands using [N-methyl-^{3}H]-scopolamine ([^{3}H]-NMS) to label M_2 and M_3-receptors, respectively. Furthermore the binding characteristics of glycopyrrolate in human peripheral lung and human airway smooth muscle preparations have also been evaluated. In all experiments, the commonly used muscarinic receptor antagonist ipratropium bromide was included as a reference drug.

Methods

Guinea-pig trachea

Tissue preparation Male Dunkin-Hartley guinea-pigs (250 – 500 g) were killed by cervical dislocation. The trachea and lungs were rapidly excised and the trachea placed in oxygenated Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6. The trachea was open longitudinally by cutting through the cartilage. The epithelium was removed by careful rubbing, minimizing damage to the smooth muscle. Indomethacin (10 μM) was present throughout the studies to inhibit the production of endogenous prostanoids.

Method Eight transverse segments, each containing three to four cartilaginous strips, were prepared and suspended between parallel wire field electrodes in 10 ml organ baths containing Krebs-Henseleit solution (pH 7.4), which continually gassed by a 95% O_2/5% CO_2 mixture and maintained at 37°C. The tissues were allowed to equilibrate for 1 h with frequent washing, under a resting tension of 1.0 g for tracheal strips, which was found to be optimal for determining changes in tension. Before the experiment, capsaicin (10 μM) was introduced and washed out 30 min after the pre-treatment to deplete endogenous tachykinins. Tissues were also pre-treated with propranolol (1 μM) 10 min before the experiment to prevent any effects of endogenous catecholamines. Isometric tension responses were measured using force-displacement transducers (Model FT-03; Grass Instruments Co., Quincy, MA, U.S.A.) connected to a polygraphy (Model 7D; Grass Instruments Co.). Electrical field stimulation (EFS) was delivered *via* two platinum wire field electrodes inserted in parallel (10 mm apart) with the tissue sample between them. A stimulator (Model D345; Digitimer Ltd., Welwyn Garden City, Hertfordshire, U.K.) provided biphasic square wave impulses with a supramaximal voltage of 40 V at source and 0.5 ms duration.

Protocol In order to elicit cholinergic contractile responses EFS was applied for 15 s periods every 4 min at a frequency of 4 Hz which produced approximately 50% of the maximal neural contraction. After at least four stable responses of equal magnitude were obtained test antagonists were introduced and further stimulations were delivered until the maximal effects of drugs were observed. Appropriate time controls were carried out for all kinetic studies. Some preparations were incubated with the minimal concentration of antagonist that gave maximal inhibition, and then the antagonist was washed out. Further stimulations were then applied until the responses returned to 50% of the original response.

Human tissue

Tissue preparation We studied tracheal strips and bronchial rings (5 – 8 mm diameter) from 15 recipients and 11 donor patients for heart and heart-lung transplantation (15 – 58 years, eight males). The lung tissues were immediately placed into oxygenated Krebs-Henseleit solution, cooled to 4°C and transported to the laboratory. The airways were dissected from the parenchyma, with removal of all bronchial blood vessels and the epithelial layer. Tissues were suspended in organ baths as with the guinea-pig tissues and allowed to equilibrate for 2 h with washing every 20 min. The resting tension was adjusted to 2 g, which was found to be optimal for measuring changes in tension in these airways. Indomethacin (10 μM) and propranolol (1 μM) were present throughout.

Protocol EFS was delivered with 40 V, 0.5 ms duration and a frequency of 8 Hz for 15 s in every 4 min (which produced approximately 50% of the maximal neural contraction). After at least four stable responses of equal magnitude were obtained, test antagonists were introduced, and further stimulations were delivered until the maximal effects of the drugs were observed. In some preparations, the concentration of antagonist used was the minimum concentration required to induce complete inhibition. When complete inhibition was achieved, the antagonist was washed out, and then further stimulations were applied until the response returned to 50% of the baseline values. Appropriate time controls in the absence of antagonists were also studied. Contractile responses obtained to EFS under these conditions were completely blocked by both atropine (1 μM) and tetrodotoxin (1 μM) indicating that the responses were evoked by stimulation of cholinergic nerves.

Radioligand binding studies

Membrane preparation All membrane preparation procedures were performed at 4°C. Human peripheral lung and airway smooth muscle, and Wistar rat heart, cerebral cortex and salivary glands were finely minced with scissors and homogenized in 10 – 20 volumes (w v^{-1} of ice-cold 0.35 M sucrose – 25 mM Tris-HCl with an Ultra-Turax homogenizer (10 – 15 bursts). After centrifugation at 800 ×*g* for 10 min at 4°C, the pellets were suspended, homogenized and re-sedimented at the same speed. The supernatants from these two stages were pooled, diluted with 50 volumes of ice-cold 25 mM Tris buffer (pH 7.4). The membranes were pelted by a centrifugation at 40,000 ×*g* for 20 min. The resulting pellets were resuspended in an appropriate volume of buffer. The membrane suspension was then stored as aliquots at −80°C. Protein concentration was determined according to the method of Lowry *et al.* (1951).

Binding assays All binding studies were performed in 1 ml of 25 mM Tris buffer (pH 7.4) containing the following tissue

concentrations (μg protein ml^{-1}: cerebral cortex, 100–200; heart, 200–400; salivary glands, 100–200; human peripheral lung 400–600 and human airway smooth muscle, 250–350. In competition experiments using [^{3}H]-NMS, a fixed concentration of 0.3 nM was used while in studies involving [^{3}H]-telenzepine, a final radioligand concentration of 0.4 nM was used. [^{3}H]-antagonist saturation curves were performed using a concentration range varying from 0.04 to 6 nM. Non-specific binding was defined as the binding in the presence of 1 μM atropine. Incubations were performed at 30°C for 2.5–3 h and terminated by rapid vacuum filtration over 0.2% polyethyleneimine pre-treated Whatman GF/C glass fibre filters using a Brandel cell harvester. The filters were washed twice with 4 ml of ice-cold Tris buffer and placed in vials with 4 ml of scintillation cocktail (Filtron X, National Diagnostics, Manville, NJ, U.S.A.) and counted in a liquid scintillation counter (Packard 2200 CA model, Meriden, CT, U.S.A.).

Kinetics studies To investigate the reversibility of the muscarinic antagonist binding, aliquots of human airway smooth muscle membranes were treated for 3 h at 30°C with assay buffer (control), glycopyrrolate (30 nM) or ipratropium bromide (30 nM). The concentration of the antagonists used in this set of experiments was calculated to occupy 80–90% of receptors. After this preincubation period, membranes were diluted (1 : 70) with assay buffer containing [^{3}H]-NMS (final concentration of 0.5 nM) in order to induce dissociation of the antagonists. [^{3}H]-NMS binding was measured at various time intervals. Non-specific binding was defined as the binding in the presence of 1 μM atropine. Specific [^{3}H]-NMS binding in the presence of the antagonists was corrected for protein concentration and expressed as a percentage of that obtained for binding to control membranes.

Statistical analysis

Contractile responses were expressed as absolute changes in tension. In kinetic studies the relevant time control in the absence of antagonist was used to control for decreased responses with time. Potency values for the test antagonists were expressed as the concentration required to inhibit cholinergic neural responses by 50% (IC$_{50}$) and calculated by a non-linear regression iterative curve fitting using Graphpad Inplot (Graphpad Inc., San Diego, CA, U.S.A.). The time of onset ($t_{1/2}$ [onset]) was defined as the time from starting the administration of the test antagonist to the attainment of 50% inhibition of cholinergic neural responses. The time for offset of action ($t_{1/2}$ [offset]) was defined as the time from washout of the test antagonist to attainment of 50% recovery of cholinergic responses. Values of ($t_{1/2}$ [onset]) and ($t_{1/2}$ [offset]) were determined by interpolation from each time-effect curve and expressed as mean $\pm$ s.e.mean.

Data for the saturation and inhibition binding studies were analysed using the non-linear regression analysis (LIGAND program) as previously described (Haddad *et al.*, 1994a,b). The precision of fit to a one- or two-site model was determined with an F test ($P < 0.01$), by comparing the residual sum of squares for fitting data to a one- or two-site model.

Drugs and chemicals

The following drugs were used: indomethacin, tetrodotoxin, atropine sulphate, ipratropium bromide, capasaicin (Sigma Chemical Co., Poole, U.K.); propranolol hydrochloride (Imperial Chemical Industries, plc., Alderley Edge, U.K.); glycopyrrolate a gift from Muro Pharmaceuticals (Muro

Pharmaceticals Inc., Tewksbury, MA, U.S.A.). [^{3}H]-NMS (specific activity 84 Ci mmol^{-1}) and [^{3}H]-telenzepine (specific activity 76.4 Ci mmol^{-1} were purchased from NEN (DuPont New England Nuclear, Stevenage, Herts, U.K.). Krebs-Henseleit solution was made up fresh every day. Indomethacin was made up in alkaline phosphate buffer (pH 7.8) of the following composition (mM): KH$_2$PO$_4$ 20 and Na$_2$HPO$_4$ 120. Drug additions did not exceed 1% of the bath volume. All concentrations refer to the final bath concentrations.

Results

Functional characteristics of glycopyrrolate in guinea-pig trachea

Potency of antagonists in inhibiting contractile responses to EFS Glycopyrrolate inhibited contraction induced by EFS (40 V, 0.5 ms, 4 Hz for 15 s every 4 min) in guinea-pig trachea in a concentration-dependent manner (IC$_{50}$ = 0.15 nM, $n = 5$–12). Glycopyrrolate was more potent than ipratropium bromide (IC$_{50}$ = 0.58 nM, $n = 6$) (Figure 1).

Time course for antagonists for inhibition of cholinergic contractile responses Glycopyrrolate and ipratropium bromide had a very similar onset of action. The $t_{1/2}$ (onset) for glycopyrrolate (10 nM) was 9.0 ± 1.7 min ($n = 8$) compared to that for ipratropium bromide (10 nM), 7.6 ± 1.2 min ($n = 5$) (Figure 2a). After washout, the inhibitory effect of ipratropium bromide (10 nM) on cholinergic responses was significantly pronounced ($t_{1/2}$ [offset] = 81.2 ± 3.7 min, $n = 5$) compared to glycopyrrolate (10 nM) ($t_{1/2}$ [offset] = 26.4 ± 0.5 min, $n = 3$) (Figure 2b).

Functional characteristics of glycopyrrolate in human airways

Potency of antagonists in inhibiting contractile responses to EFS Glycopyrrolate inhibited contraction induced by EFS (40 V, 0.5 ms, 8 Hz for 15 s every 4 min) in human trachea in a concentration-dependent manner (IC$_{50}$ = 0.44 nM, $n = 3$–8). Glycopyrrolate was more potent than ipratropium bromide (IC$_{50}$ = 1.36 nM, $n = 4$–7) (Figure 3).
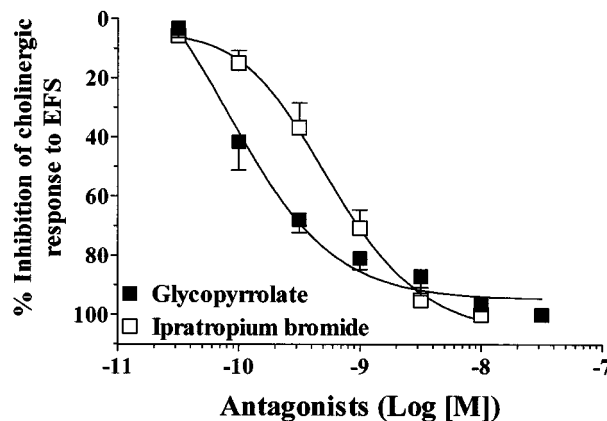

Figure 1 Effects of glycopyrrolate and ipratropium bromide on constrictor responses induced by electrical field stimulation (4 Hz, 40 V, 0.5 ms for 15 s every 4 min) in guinea-pig trachea. Each value is the mean $\pm$ s.e.mean of 5–12 animals.

Time course for antagonists for inhibition of cholinergic contractile responses Glycopyrrolate and ipratropium bromide had a very similar onset of action. The $t_{1/2}$ (onset) for

glycopyrrolate (3 nM) was 19.7 ± 3.5 min ($n = 9$) compared to that for ipratropium bromide (10 nM), 14.9 ± 2.8 min ($n = 6$) (Figure 4a). After washout, the inhibitory effect of glycopyrrolate (3 nM) on cholinergic responses was significantly prolonged ($t_{1/2}$ [offset] < 96 min, $n = 6$) compared to ipratropium bromide (10 nM) $Tt_{1/2}$ [offset] $= 59.1 \pm 17.8$ min, $n = 3$) (Figure 4b).

Binding characteristics of glycopyrrolate

Selectivity of glycopyrrolate for $M_1 - M_3$ receptors The pharmacological profile of glycopyrrolate was determined in rat cerebral cortex using [³H]-telenzepine to label M_1-receptors and in rat heart and salivary glands using [N-methyl-³H]-scopolamine ([³H]-NMS) to label M_2- and M_3-receptors, respectively. Specific [³H]-NMS binding to muscarinic receptors in rat heart and salivary glands was saturable and best described by the interaction of the radioligand with a single population of high affinity binding sites. The affinities (K_D in nM) were 0.09 ± 0.02 and 0.35 ± 0.04 in rat salivary glands and heart, respectively (data not shown). In rat cerebral cortex, [³H]-telenzepine, at a concentration of 0.4 nM, labelled exclusively the M_1-receptors (Schudt *et al.*, 1989). [³H]-Telenzepine/telenzepine displacement studies revealed a K_D value of 0.62 nM. Ipratropium bromide bound to all the receptor subtype studied
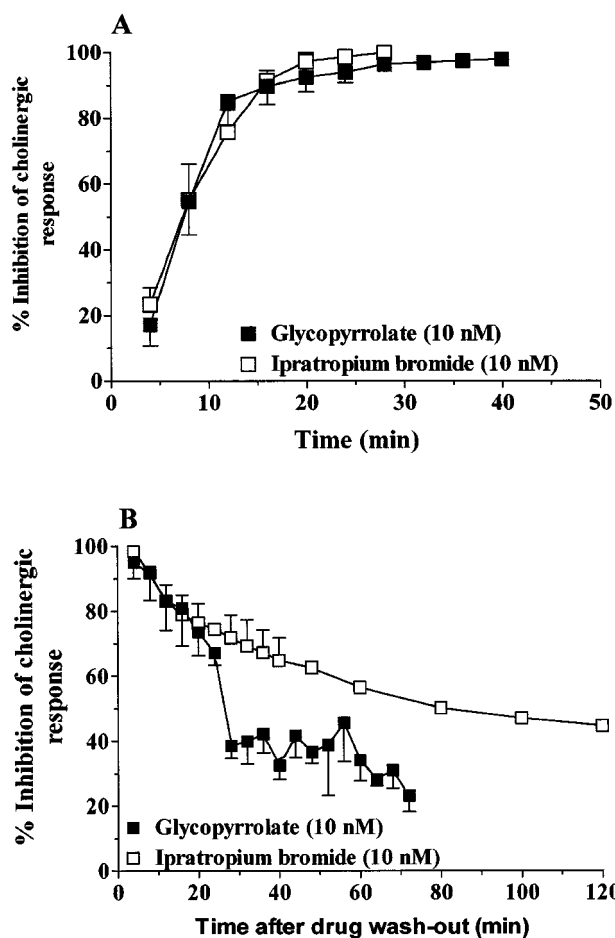
Figure 2 (A) Time course of onset of action of glycopyrrolate and ipratropium bromide in guinea-pig trachea. Each value is the mean $\pm$ s.e.mean of 5–8 animals. (B) Time course of the effect of glycopyrrolate and ipratropium bromide after washout of the test drugs in guinea-pig trachea. Each value is the mean $\pm$ s.e.mean of 3–5 animals.
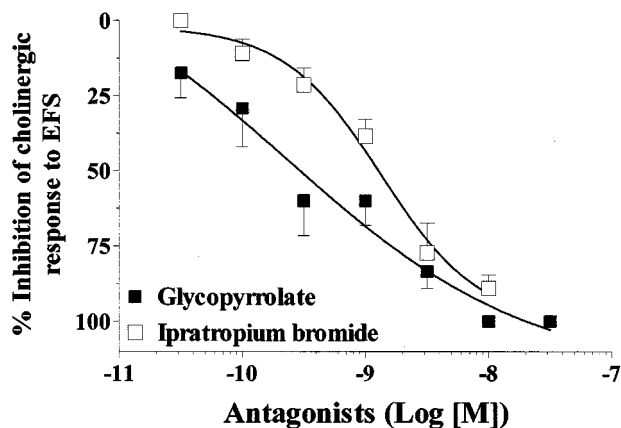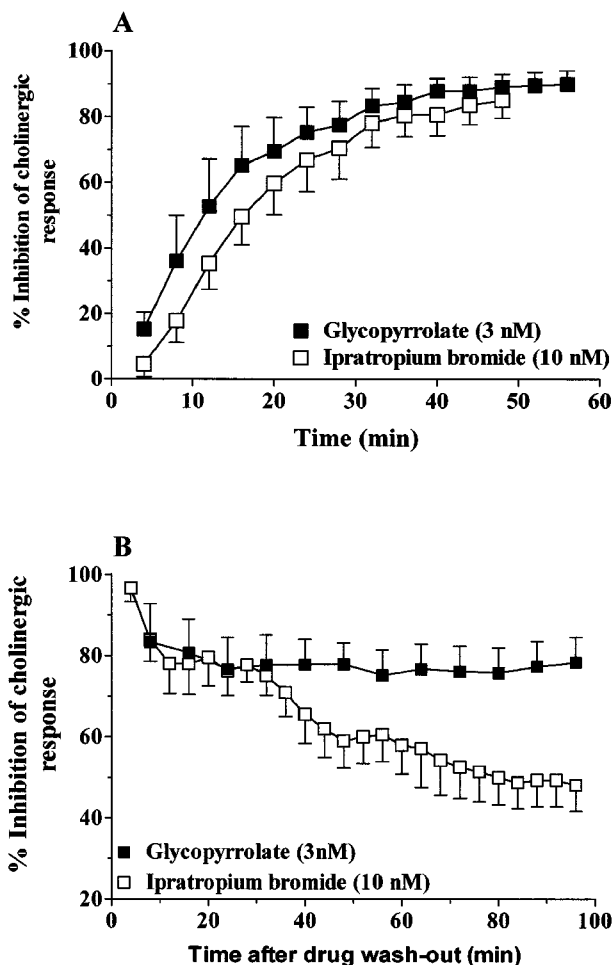
Figure 3 Effects of glycopyrrolate and ipratropium bromide on constrictor responses induced by electrical field stimulation (8 Hz, 40 V, 0.5 ms for 15 s every 4 min) in human airways. Each value is the mean $\pm$ s.e.mean of 3–8 patients.

Figure 4 (A) Time course of onset of action of glycopyrrolate and ipratropium bromide in human airways. Each value is the mean $\pm$ s.e.mean of 6–9 patients. (B) Time course of effects of glycopyrrolate and ipratropium bromide after washout of the test drugs in human airways. Each value is the mean $\pm$ s.e.mean of 3–6 patients.

with almost the same high affinity in agreement with published data. Similar to ipratropium bromide, glycopyrrolate showed no selectivity in its interaction with M_1, M_2 and M_3-receptors, although a 3–5-fold higher affinity at M_3-receptors compared to M_1 and M_2-receptors was observed (Figure 5, Table 1).

Affinity of glycopyrrolate and ipratropium bromide in human airways   The non-selective and hydrophilic muscarinic antagonist [³H]-NMS was used to label muscarinic receptors present in human peripheral lung and airway smooth muscle homogenates. Specific [³H]-NMS binding was saturable and best described by the interaction of the radioligand with a single and homogeneous population of high affinity binding sites. The affinites (K_D in nM) were 0.21 ± 0.02 and 0.34 ± 0.08 in human long and human airway smooth muscle, respectively. In competition studies, glycopyrrolate and ipratropium bromide fully inhibited the specific binding of [³H]-NMS in a concentration dependent manner in both preparations (Figure 6a, b). The results of the competition studies are shown in Table 2.

Kinetics profile of glycopyrrolate and ipratropium bromide in human airway smooth muscle   Wash-out experiments suggest that glycopyrrolate possesses a longer duration of action when

compared to ipratropium bromide (Figure 4b). To investigate the potential mechanisms by which glycopyrrolate achieves its duration of action *in vitro*, we assessed its rate of dissociation from human airway smooth muscle muscarinic receptors indirectly by monitoring the rate of [³H]-NMS association. Human airway smooth muscle membranes were preincubated for 3 h with glycopyrrolate (30 nM) or ipratropium bromide
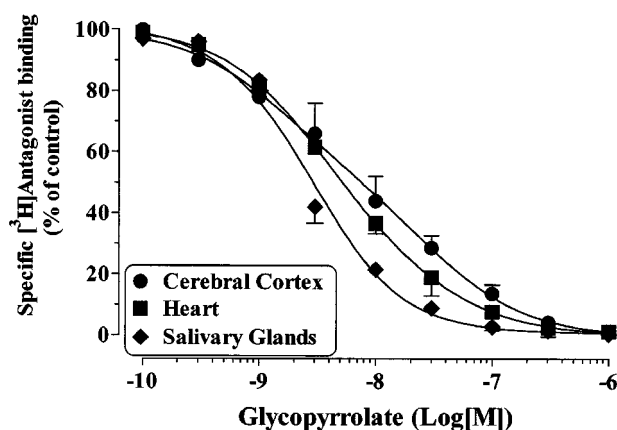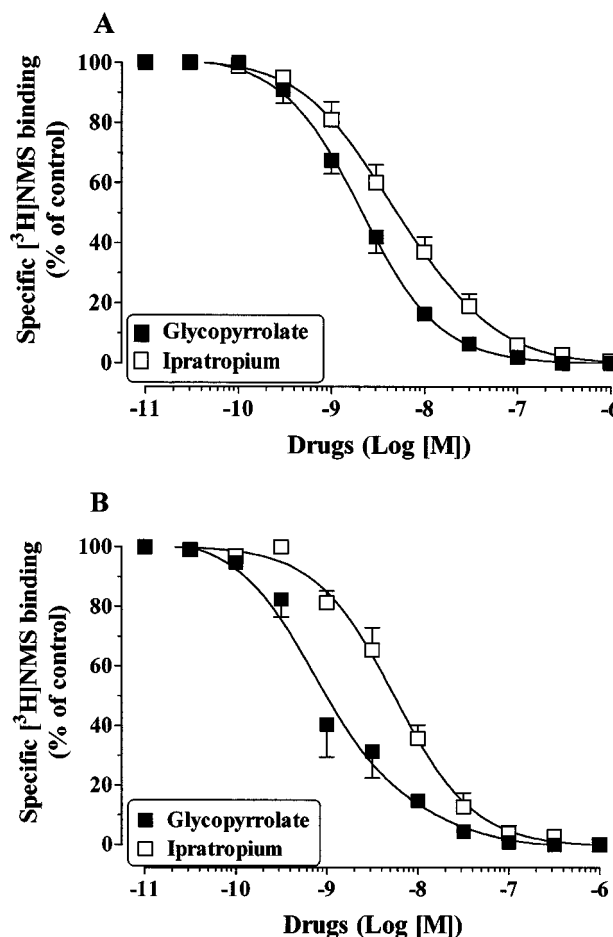

Figure 6   Inhibition of [³H]-NMS binding by glycopyrrolate and ipratropium bromide in human peripheral lung (A) and human airway smooth muscle (B). Values are means±s.e.mean from 3–6 independent experiments performed in duplicate on separate membrane preparations.

Figure 5   Inhibition of [³H]-NMS (rat heart and salivary glands) and [³H]-telenzepine (rat cerebral cortex) binding by glycopyrrolate. Values are means±s.e.mean from 4–6 independent experiments performed in duplicate on separate membrane preparations.

Table 1   Inhibition of [³H]-telenzepine (cerebral cortex) and [³H]-NMS (heart and salivary glands) binding by glycopyrrolate and ipratropium bromide

	Cerebral cortex (M_1)	Heart (M_2)	Salivary glands (M_3)
Glycopyrrolate			
K_i (nM)	2.38 ± 0.71	3.72 ± 0.86	0.61 ± 0.06
n_H	0.87 ± 0.09	0.96 ± 0.05	1.00 ± 0.07
Ipratropium bromide			
K_i (nM)	1.24 ± 0.36	3.54 ± 1.27	0.64 ± 0.06
n_H	0.92 ± 0.01	0.99 ± 0.04	1.01 ± 0.02

Inhibitory dissociation constants (K_i) values (in nM) and Hill coefficients (n_H) were determined from competitive binding curves. Values are mean±s.e.mean of 4–6 independent experiments performed in duplicate on separate membrane preparations.

Table 2   Binding characteristics of glycoprrolate and ipratropium bromide in human airways

	Peripheral lung	Airway smooth muscle
Glycopyrrolate		
K_i (nM)	0.74 ± 0.10	$0.69+0.11$
n_H	$1.00+0.01$	$0.92+0.06$
Ipratropium bromide		
K_i (nM)	$0.51+0.11$	$3.59+0.13$
n_H	$0.92+0.02$	0.99 ± 0.05

Inhibitory dissociation constants (K_i) values (in nM) and Hill coefficients (n_H) were determined from competitive binding curves. Values are mean±s.e.mean of 3–6 independent experiments performed in duplicate on separate membrane preparations.

(30 nM) in order to occupy 80–90% of the receptors. To induce antagonist dissociation, the volume of the membrane's suspension was then increased 70 fold with the assay buffer containing [^{3}H]-NMS, thereby reducing the concentration of both antagonists. The time course of [^{3}H]-NMS association in buffer- and ipratropium-pre-treated membranes was rapid and reached equilibrium within 30–40 min of incubation ($t_{1/2}$ ~7 min). In glycopyrrolate pre-treated membranes, the rate of [^{3}H]-NMS association (shown in Figure 7 as percentage of glycopyrrolate-occupied receptors) was considerably slower. This result suggests that [^{3}H]-NMS binding to human lung muscarinic receptors was probably limited by the slow dissociation rate of glycopyrrolate.

Discussion

In this study we examined the effect of glycopyrrolate, a muscarinic receptor antagonist, on EFS-induced cholinergic neurotransmission, but not on exogenous ACh-induced bronchoconstriction, because EFS-induced bronchoconstriction may be more representative of an increase in vagal tone than exogenous ACh-induced bronchoconstriction. We have demonstrated that glycopyrrolate was more potent (3–4 fold) than the anti-cholinergic bronchodilator commonly used in the clinic, ipratropium bromide, in inhibiting cholinergic neural responses in guinea-pig and human airways.

In guinea-pig trachea and human airways, the onset of action of glycopyrrolate ($t_{1/2}$ [onset] of 9–19.8 min) was similar to that obtained with ipratropium bromide and faster compared to tiotropium bromide (Ba 679 BR) ($t_{1/2}$ [onset] of 34–43 min) (Takahashi *et al.*, 1994). However, there is a marked difference in the dissociation profile of glycopyrrolate and ipratropium bromide in guinea-pig and human airways. In this set of experiments, airway tissue was incubated with the
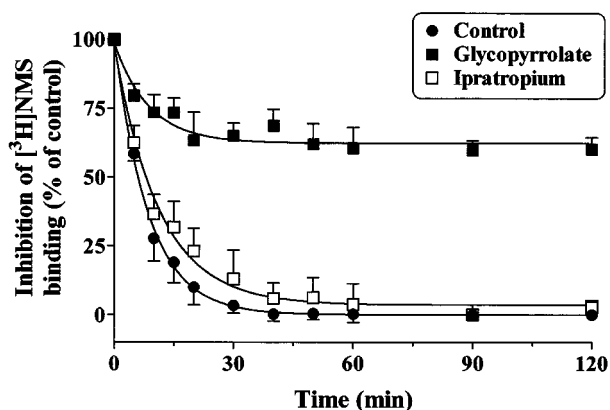

Figure 7 [^{3}H]-NMS binding to human airway smooth muscle muscarinic receptors occupied by glycopyrrolate and ipratropium bromide. Human airway membranes were treated with the assay buffer (Control) or the test antagonists (each at 30 nM) for 3 h at 30°C. After this preincubation period, the volume of membrane suspension was then diluted (in order to induce antagonist dissociation) with the assay buffer containing final [^{3}H]-NMS concentration of 0.5 nM. The association of [^{3}H]-NMS (expressed as percentage inhibition of [^{3}H]-NMS binding which reflect the percentage of glycopyrrolate or ipratropium occupied receptors) was measured at appropriate time intervals. Specific [^{3}H]-NMS binding in the presence of the antagonists was corrected for protein concentration and expressed as a percentage of that obtained for binding to control membranes. Values are means ± s.e.mean from three independent experiments performed in duplicate on separate membrane preparations.

minimal concentration of the test antagonist that produced complete inhibition of the cholinergic neural response. When complete inhibition was achieved, the antagonist was washed out, and then further stimulations were applied until the response returned to 50% of the baseline values. The time for offset of action ($t_{1/2}$ [offset]) was defined as the time from washout of the test antagonist to attainment of 50% recovery of cholinergic responses. In guinea-pig trachea, the inhibitory effect of glycopyrrolate (10 nM) and ipratropium bromide (10 nM) on cholinergic responses was similar with time, although the time taken for the offset of action of glycopyrrolate was less than that obtained with ipratropium bromide. After wash-out, the inhibitory effect of glycopyrrolate (3 nM) was significantly more prolonged (over 70% inhibition of the neural response) compared to ipratropium bromide (10 nM) in human tissues. In fact, a $t_{1/2}$ [offset] could not be obtained for glycopyrrolate within the time our preparations were maintained. Therefore, our results suggest differences in the dissociation kinetics of these antagonists with muscarinic receptors in different species. The longer duration of action of glycopyrrolate in inhibiting cholinergic neurotransmission in human airways suggests that it may have a therapeutic advantage over existing drugs such as ipratropium bromide. This property is also shared with the recently developed muscarinic antagonist tiotropium bromide. We have previously shown that this antagonist, in addition to its powerful inhibitory effect of cholinergic nerve-induced contraction of guinea-pig and human airways, dissociated extremely slowly from post-junctional muscarinic receptors when compared to atropine or ipratropium bromide (Takahashi *et al.*, 1994).

We have also determined the binding characteristics of glycopyrrolate. We first investigated the selectivity of glycopyrrolate and ipratropium bromide in their interaction with the rat cerebrocortical M_1-receptors labelled with ^{3}H-telenzepine and heart M_2- and salivary glands M_3-receptors labelled with ^{3}H-NMS. Ipratropium bromide bound to all the receptor subtypes studied with almost the same high affinity in agreement with published data. Similar to ipratropium bromide and tiotropium bromide (Ba 679 BR), glycopyrrolate showed no selectivity in its binding to the M_1–M_3-receptors (Haddad *et al.*, 1994a; Gomez *et al.*, 1995), although a 3–5 fold higher affinity at M_3-receptors compared to M_1 and M_2 receptors was observed. It should be noted that functional affinity derived from Schild analysis has shown that glycopyrrolate possesses a high affinity at M_1/M_3 (pKB values of 10.31–11) receptors compared to M_2 receptors (pA2 value of 8.16–9.09) (Lau & Szilagyi, 1992; Fuder & Meincke, 1993).

The affinity of glycopyrrolate and ipratropium bromide for muscarinic receptors in human peripheral lung and airway smooth muscle membranes was also evaluated. ^{3}H-NMS labelled homogeneous binding sites in these preparations were not discriminated by the drugs used. Indeed, both of the antagonists examined produced mass action competition curves compatible with the existence of a single receptor subtype in the different membrane preparations used. It should be noted that a 5 fold higher affinity at human airway smooth muscle muscarinic receptors was observed for glycopyrrolate compared to ipratropium bromide.

Experiments have also been performed to determine the dissociation profile of glycopyrrolate from human airway muscarinic receptors. Unlike ipratropium bromide (30 nM) which dissociated so quickly that there was little difference in the [^{3}H]-NMS association to vehicle treated membranes, glycopyrrolate (30 nM) has a strong protective effect against [^{3}H]-NMS binding (>60%) which lasted for 2 h. These results

suggest that the slow dissociation profile of glycopyrrolate from human airway smooth muscle muscarinic receptors provide a mechanistic explanation for the long duration of action of this drug observed in functional experiments. Similar data were obtained in human peripheral lung with the new muscarinic antagonist tiotropium bromide (Haddad *et al.*, 1994a). The slow dissociation profile of tiotropium bromide was in agreement with functional and clinical studies confirming its long lasting bronchodilator action in patients with asthma and COPD (O'Connor *et al.*, 1996; Barnes *et al.*, 1995).

Prolonged inhibitory effects of glycopyrrolate have also been observed in functional tests *in vitro* (this report) and *in vivo* in humans. In normal subjects, glycopyrrolate produces significant bronchodilation of long duration (Gal & Suratt, 1981; Gal *et al.*, 1984). In patients with chronic obstructive pulmonary disease, inhaled nebulized glycopyrrolate (1 mg) produced a bronchodilating effect that lasted for 8 h (Tzelepis *et al.*, 1996). Similarly, in patients with asthma, when compared to placebo, metered-dose aerosols of glycopyrrolate (doses ranging from 100 – 1200 mg) elicited significantly greater bronchodilation that was noted within 30 min of dosing and was sustained for at least 8 – 12 h (Walker *et al.*,

1987; Schroeckenstein *et al.*, 1988). Thus, there seems to be a close correlation between the binding experiments obtained in human airway smooth muscle and the functional data regarding the antagonist dissociation profile and the duration of action in humans.

Anticholinergic drugs have been found to be as effective and, in some cases more effective that β-agonists as bronchodilator agents in the treatment of airways obstruction in patients with COPD (Gross, 1988) and in some patients with nocturnal asthma (Coe & Barnes, 1986; Morrison *et al.*, 1988). However, the duration of action of the present anti-cholinergic drugs is insufficient to provide convenient maintenance therapy for the patients or to control nocturnal asthma. These results suggest, that at least in human airways, glycopyrrolate had a longer duration of action than ipratropium bromide and that it may prove a more efficient therapy for these conditions than the drugs commonly used today.

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