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Pharmacological assessment of the duration of action of glycopyrrolate vs tiotropium and ipratropium in guinea-pig and human airways

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1 Our study was aimed at investigating the duration of the bronchodilator action of the antimuscarinc drug glycopyrrolate compared to tiotropium and ipratropium.

2 In the guinea-pig isolated trachea, the time ($t_{1/2}$) necessary for a contractile response to carbachol (0.3 μM) to return to 50% recovery after washout of the antagonist was studied. The offset of the antagonist effect of glycopyrrolate, tiotropium and ipratropium (10 nM each) was $t_{1/2} = 4.0 \pm 0.5$, >4.5 and 0.5 ± 0.1 h, respectively. At 4.5 h from the washout of the antagonist, the recovery of the response to carbachol was 50 ± 8, 10 ± 4 and 70 ± 7%, respectively.

3 In the human isolated bronchus, the offset of the bronchodilator effects of glycopyrrolate (3 nM), tiotropium (1 nM) and ipratropium (10 nM) was $t_{1/2} = 3.7 \pm 0.2$; >6 and 3.0 ± 0.2 h, respectively. At 6.0 h from the washout of the antagonist, the recovery of the response to carbachol (1 μM) was 101 ± 10, 27 ± 3 and 110 ± 10%, respectively.

4 In anaesthetized guinea-pigs, acetylcholine-induced bronchoconstriction was markedly reduced by intratracheal instillation of glycopyrrolate (3 nmol kg^{-1}; 88.1 ± 4% inhibition), tiotropium (1.3 nmol kg^{-1}; 86.2 ± 5% inhibition) or ipratropium (1.45 nmol kg^{-1}; 88.1 ± 10% inhibition). These inhibitory effects assessed 3 or 24 h after antagonist administration were reduced to 69.9 ± 5 and 29.7 ± 6%; 28.3 ± 5 and 14.2 ± 5% for glycopyrrolate and ipratropium, respectively, whereas they remained stable (83.5 ± 4; 70.6 ± 6) for tiotropium. The residual inhibitory effect of glycopyrrolate was also assessed at 16 h from administration, and proved to be as low as that found at 24 h (31.2 ± 10 vs 29.7 ± 6%, respectively).

5 In conclusion, glycopyrrolate-induced bronchodilation has a longer duration than that of ipratropium, but less than that of tiotropium. The efficacy of a possible glycopyrrolate-based therapy for asthma or chronic obstructive pulmonary disease given once-a-day is not guaranteed by the present investigation.

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Abbreviations: ACh, acetylcholine; COPD, chronic obstructive pulmonary disease

Introduction

Chronic obstructive pulmonary disease (COPD) is a syndrome characterized by progressive airflow limitation caused by chronic inflammation of the airways and lung parenchyma, which is caused predominantly by chronic cigarette smoking. The current treatment of COPD is palliative, as there is no available therapy capable of halting the decline in lung function and the parenchymal destruction associated with the disease (Krishna *et al*., 2004; Belvisi *et al*., 2004 for a review). Bronchodilators are the mainstay therapy in COPD, as they increase expiratory flow by decreasing airway smooth muscle tone, thus leading to enhanced lung emptying. In asthma and COPD, bronchoconstriction and mucus secretion are increased and the airways become hyperresponsive to contractile agents. In COPD, these changes are caused mostly by increased parasympathetic nerve activity. Acetylcholine (ACh) released from parasympathetic nerves activates postjunctional muscarinic M_3 receptors present on airway smooth muscle and submucosal glands to produce bronchoconstriction and mucus secretion, respectively. ACh also feeds back onto prejunctional muscarinic M_2 receptors to inhibit further ACh release (Haddad & Rousell, 1998; Barnes, 2004b).

Anticholinergics are the most effective class of bronchodilators in COPD, more effective than β_2 receptor agonists which, in contrast, are very potent in asthma where bronchoconstriction is sustained by a variety of mediators (Gross & Skorodin, 1984; Rennard *et al*., 1996; Barnes 2004a). Anticholinergics that are used more frequently in COPD are quaternary ammonium salts like ipratropium bromide (Gross, 1988), oxitropium bromide (Skorodin *et al*., 1986) and

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tiotropium bromide (Disse *et al*., 1993). The introduction of tiotropium bromide has represented a breakthrough in the pharmacological management of the COPD. Tiotropium bromide possesses higher affinity for muscarinic receptors than ipratropium and, more importantly, it dissociates very slowly from M_3 muscarinic receptors (Disse *et al*., 1993; Haddad *et al*., 1994), a behavior thought to be responsible for the prolonged action of tiotropium. Accordingly, high potency and long-lasting duration of action of tiotropium were demonstrated in functional studies conducted in guinea-pig and human isolated airways (Takahashi *et al*., 1994) and in anaesthetized dogs (Disse *et al*., 1993). As a consequence, the long duration of action of tiotropium allows for once-a-day administration of this drug in human therapy (Littner *et al*., 2000; Gross, 2004). Another well-known quaternary ammonium compound endowed with potent antimuscarinic activity is glycopyrrolate. Glycopyrrolate is currently used as gastric antisecretory agent, to reduce salivary secretions, and in preanaesthetic medication (Mirakhur & Dundee, 1981; Muir & von Gunten, 2000; Tscheng, 2002). In addition, pilot clinical studies have shown that inhaled glycopyrrolate displays bronchodilator activity in asthmatic and COPD patients, an effect apparently lasting for (at least) 8–12 h (Walker *et al*., 1987; Schroeckenstein *et al*., 1988; Tzelepis *et al*., 1996). However, limited preclinical information is available concerning the duration of action of glycopyrrolate as a bronchodilator, thus it is difficult to estimate whether a future glycopyrrolate-based therapy for COPD could be administered once-daily, or instead would require more frequent administrations. Indeed, in the sole preclinical evaluation of the duration of antibronchospastic action of glycopyrrolate, performed in guinea-pig and human isolated airways, the inhibitory effects produced by this antagonist were studied for only 100–120 min (Haddad *et al*., 1999). Thus, in the present investigation we have studied the duration of action of the bronchodilator activity of glycopyrrolate in comparison with tiotropium and ipratropium up to 4.5–6.0 h from its administration in the isolated guinea-pig trachea and human bronchus, and up to 24 h in guinea-pig *in vivo*.

Methods

In vitro *studies: guinea-pig isolated trachea*

All the experiments were performed according to Haddad *et al*. (1999), with modifications. Briefly, the whole trachea was excised from male albino Dunkin–Hartley guinea-pigs (450–550 g, Charles River Laboratories Italia, Calco, Italy), opened along a longitudinal section opposite to the smooth muscle, and cut into two transverse preparations (4–5 zigzag segments each). Each preparation was placed in 20-ml organ bath filled with oxygenated (O_2 95% and CO_2 5%) normal Krebs–Henseleit solution and maintained at 37°C. Tracheal preparations were connected to isometric force transducers under a resting tone of 10 mN. Data were recorded by means of a PowerLab system (www.ADinstruments.com). After an equilibration period of 60 min, carbachol was administered to preparations at a concentration (0.3 μM) that in preliminary experiments had been shown to produce 80–90% of maximal contraction. At steady tonic contraction, preparations were relaxed by isoprenaline (0.3 μM). After a thorough washout, carbachol (followed by isoprenaline) was administered again, until a reproducible contractile response was obtained. Isoprenaline-induced relaxation was used as the maximal inhibitory reference effect. At this point the test compounds were assayed, as described below.

In vitro *studies: human isolated bronchus*

Specimens of human lung were obtained from six patients (four males) age 48–66 years, undergoing surgery for lung cancer. Ethical approval for the experiments performed on human tissue was obtained from the Ethics Committee of the University of Florence. No patient received radio- or chemotherapy before the operation. In all patients, preanaesthetic medication included intramuscular atropine (1 mg) and diazepam (10 mg). Anaesthesia was induced by sodium thiopental (500 mg intravenous (i.v.)) and maintained with N_2O/O_2 (1/2) and halothane (0.6–1%). The patients received pancuronium bromide (6 mg i.v.) during induction of anaesthesia. Immediately after the surgical removal of the lung tissue, bronchial rings of about 1–5 mm (internal diameter) were excised from the parenchyma, deprived of bronchial blood vessels and carefully rubbed with a cotton-tip applicator in order to remove the epithelium. All specimens appeared macroscopically normal without signs of tumor or inflammation. The bronchial rings were rapidly placed in ice-cold gassed (96% O_2 and 4% CO_2) Krebs–Henseleit solution, overnight. The next day, 15–20 h after excision, the rings were placed in 5-ml organ baths filled with oxygenated Krebs–Henseleit solution at 37°C, under a resting tension of 20 mN. Changes in basal tone were recorded isometrically. The experiments commenced after a 180-min equilibration period. Carbachol was administered twice to preparations at a concentration (1 μM) that in preliminary experiments had been shown to produce 80–90% of maximal contraction. At steady tonic contraction, preparations were relaxed by isoprenaline (0.3 μM). After a thorough washout and a 60 min stabilization period, the test compounds were analyzed, as reported below.

In vitro *studies: estimate of antagonist potency and offset of the inhibitory effect produced by test compounds*

In preliminary experiments, pretreatment with tiotropium produced a nonparallel rightward shift of the concentration–response curve to carbachol in guinea-pig trachea and human bronchus and depressed E_{max} (data not shown), according to its known slow reversible interaction with muscarinic receptors. As a pA_2 value could not be estimated with tiotropium, we tested all compounds for their ability to reverse an M_3 receptor-mediated contraction. Thus, a cumulative concentration–response curve to test antagonists was constructed on guinea-pig or human preparations precontracted with carbachol, until a complete inhibition of smooth muscle tone was achieved. As the onset of action of tiotropium at M_3 receptors was shown to be very slow (Takahashi *et al*., 1994), we allowed tiotropium (and the other antagonists) to take all the time necessary to produce steady-state responses during construction of the concentration–response curves. Usually a curve to tiotropium took 2.5–3.0 h in the guinea-pig trachea, and 2.0–2.5 h in the human bronchus. During these periods of time the contractile response to carbachol remained stable (loss of tension $\leqslant$10%) in both tissues. The antagonist concentration

producing a 50% reversal of carbachol-induced tonic contraction (IC_{50}) was taken as a measure of antagonist potency in both bioassays. In the experiments aiming at assessing the offset of the inhibitory effects produced by test compounds, the minimal concentration of each test antagonist known to produce a (almost) maximal inhibitory effect was administered to carbachol-precontracted preparations. As soon as carbachol-induced tonic contraction was reversed by the antagonist, the organ bath solution was renewed and preparations were thoroughly washed with fresh Krebs–Henseleit solution. Tiotropium, glycopyrrolate and ipratropium were washed out only after a complete and sustained relaxation of the smooth muscle was achieved. Usually the time taken by tiotropium in these latter experiments was 1.0–1.5 h in both the guinea-pig trachea and human bronchus. Carbachol was administered again (at 45–60 min interval, between washout and next administration) during the next 4.5–6.0 h, in the guinea-pig trachea or human bronchus, respectively. A $t_{1/2}$ (offset) value, that is the time taken for response to return to 50% recovery after washout of the test antagonist, was estimated whenever possible. In addition, the percentage of response to carbachol obtained 4.5–6.0 h after the washout of the test antagonist was taken as a measure of the offset (reversibility) of the compound under investigation. Control matched preparations receiving the vehicle of the test antagonists were used to assess the reproducibility of the response to carbachol in the absence of M_3 receptor blockade.

In vivo *study: ACh-induced bronchoconstriction in anaesthetized guinea-pig*

Male albino Dunkin–Hartley guinea pigs (450–550 g, Charles River Laboratories Italia, Calco, Italy) were used for all the experiments. Animals were anaesthetized with sodium pentobarbital (90–100 mg kg^{-1}, intraperitoneally (i.p.)). The carotid artery and jugular vein were cannulated to monitor systemic blood pressure and for drug administration, respectively. In order to maintain a stable level of anaesthesia throughout the duration of the experiment, sodium pentobarbital (10 mg kg^{-1} h^{-1}) was continuously infused into the carotid artery. Body temperature was kept constant at 37°C by a heated blanket.

The trachea was cannulated and the lungs were ventilated artificially with a small animal constant volume ventilator (Harvard Apparatus, South Natick, Massachusetts, U.S.A.) at a frequency of 70 strokes min^{-1} and at a tidal volume of 10 ml kg^{-1}. To avoid spontaneous breathing, the animals were injected intravenously (i.v.) with pancuronium bromide (2 mg kg^{-1}). Bronchoconstriction was induced by i.v. injection of ACh, 20 μg kg^{-1}. In control experiments, repeated injections of ACh produced reproducible short-lasting (1–2 min duration) bronchoconstrictions. Bronchoconstriction, quantified as a reduction of tidal volume, was evaluated according to the method of Konzett & Roessler (1940). Systemic blood pressure and changes in airway resistance were monitored on a two-channel pen recorder (Ugo Basile, Comerio, Italy).

Following stabilization of the artificial breathing and blood pressure, animals were injected (i.v.) with ACh every 3 min, until three stable and reproducible basal responses were obtained. Test compounds were administered locally: 100 μl of solution was instilled intratracheally, directly through the tracheal cannula. Test compound instillation was immediately followed by two consecutive insufflations of air (3 ml each), in order to facilitate the distribution of the liquid solution into the airways (Lundberg & Saria, 1983). ACh challenge was then repeated at 5, 15, 30 min and then every 30 min up to 180 min from the intratracheal administration of test compounds. The effect of test compounds was expressed as percentage of inhibition of ACh-evoked bronchoconstriction, as compared to basal response.

In order to assess the residual inhibitory activity of test compounds 16–24 h after their administration, a modified protocol was adopted, as follows. The day before the challenge animals were anaesthetized with isoflurane and cannulated by a laryngoscope to expose the trachea. Test compounds (or vehicle) were then instilled locally as described above. Guinea-pigs were then allowed to recover from anaesthesia and fed normally. The day after, animals were anaesthetized with sodium pentobarbital (90–100 mg kg^{-1}, i.p.) and surgically prepared as described previously. ACh (20 μg kg^{-1} i.v.) was administered at 3 min intervals, until three stable bronchoconstrictor responses were obtained. The number of ACh challenges never exceeded 10. At the end of the experiment, the animals were challenged with histamine (5 mg kg^{-1} i.v.) in order to verify bronchial reactivity to a contracting agent. Time-matched, vehicle-treated, animals were used as controls. Ethical approval of the experimental protocols with animals was obtained from the local Ethics Committee.

Statistical analysis

All values in the text, table and figures are expressed as means ± s.e.m. of the given number (n) of experiments. Statistical analysis was performed using Student's t-test for paired or unpaired data, or by one-way analysis of variance (ANOVA) when applicable. $P<0.05$ was considered a level of statistical significance.

Chemicals

ACh chloride, carbachol, histamine, ipratropium bromide, isoprenaline, pancuronium were from Sigma (St Louis, MO, U.S.A.). Glycopyrrolate was from Kemprotec (Middlesbrough, U.K.). Tiotropium bromide was synthesized at Chiesi Chemical Department. Stock solutions (10 mM) of ipratropium, tiotropium and glycopyrrolate were prepared in 100% DMSO. Further dilutions were prepared with saline. In *in vivo* studies DMSO final concentration in the instilled solutions never exceeded 0.3%, in saline. In control *in vitro* and *in vivo* experiments, administration of 1% DMSO in saline was ineffective *per se*.

Results

Duration of antimuscarinic activity in the guinea-pig isolated trachea

Carbachol (0.3 μM) produced prompt and sustained tonic contractions of the tracheal smooth muscle, which were highly reproducible over a period of several hours (Figures 1 and 2). In carbachol (0.3 μM)-precontracted preparations, tiotropium produced a slowly developing inhibition of smooth muscle tone, showing a potency (pIC_{50}) in the subnanomolar range

(Table 1). The maximal inhibitory effect of tiotropium was obtained at 10 nM (Figure 2a). Glycopyrrolate and ipratropium produced concentration-dependent inhibitions of carbachol-induced tonic contraction with pIC_{50} similar to that shown by tiotropium (Table 1, Figure 2a). None of the three antagonists (10 nM each) inhibited a tracheal smooth muscle tone (averaging ~85% of that produced by carbachol) induced by neurokinin A (1 μM) ($n=4$ each, data not shown).

In experiments aimed at evaluating the offset of action, tiotropium (10 nM) was administered to carbachol (0.3 μM)-precontracted preparations and left in contact with the tissue until complete inhibition was attained. After washout of tiotropium, the contractile response to carbachol (0.3 μM, given every 45 min) remained depressed over a period of 4.5 h, despite the removal of the antagonist from the buffer solution (Table 1; Figures 1 and 2b). In contrast, ipratropium (10 nM)-induced inhibition showed to be rapidly reversible ($t_{1/2}=0.5$ h; Table 1), while the offset of glycopyrrolate (10 nM)-induced inhibitory effect was intermediate between that of tiotropium and ipratropium. Indeed, 4.5 h after washout of glycopyrrolate, tracheal preparations had recovered 50% of their initial responsiveness to carbachol (Table 1; Figure 2b).

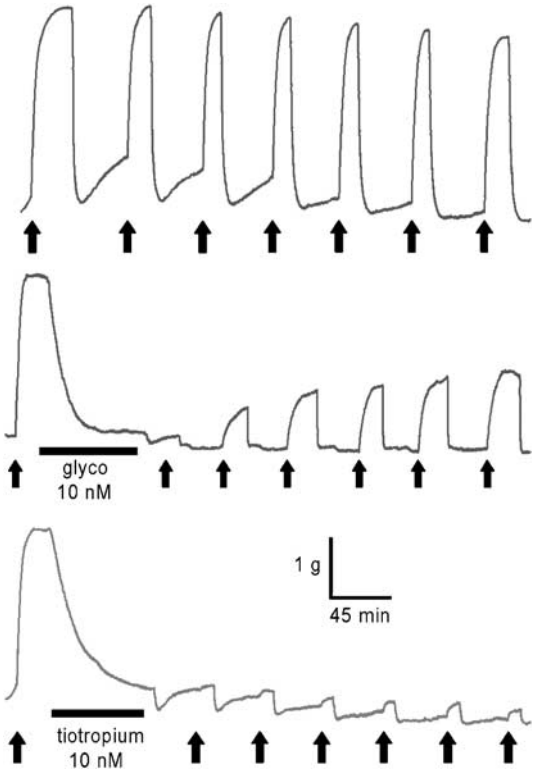

Figure 1 Typical tracings showing the rate of offset of the antibronchospastic effects produced by glycopyrrolate and tiotropium in the guinea-pig isolated trachea. Arrows indicate administration of carbachol (0.3 μM). Test antagonists were administered at a steady state on the tonic contraction produced by carbachol and, after completion of their smooth muscle relaxing effect, they were washed out from the bath. Subsequently, carbachol was administered again every 45 min for 4.5 h. The upper tracing represents a control experiment.

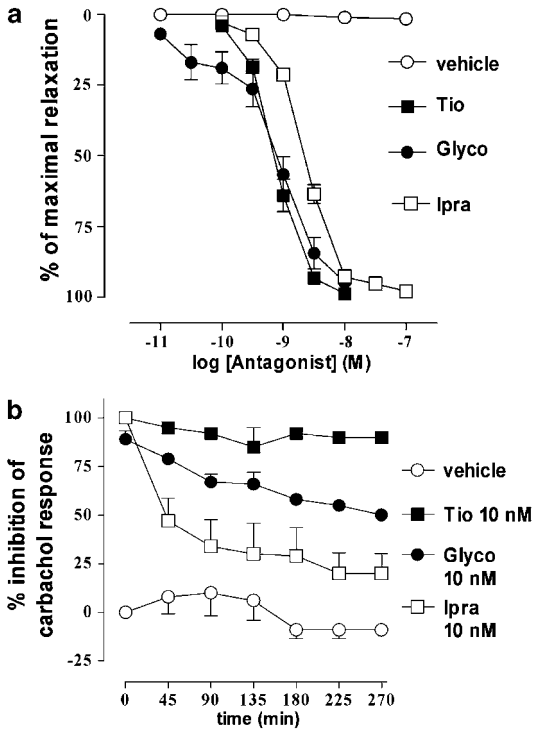

Figure 2 (a) Concentration-dependent inhibition by tiotropium (Tio), glycopyrrolate (Glyco) and ipratropium (Ipra) of carbachol (0.3 μM)-induced tonic contraction in the guinea-pig isolated trachea. (b) Decay of the inhibitory effects produced by the test compounds against contractile responses to carbachol (0.3 μM) in the guinea-pig isolated trachea. After washout of the antagonist (time = 0), carbachol was administered at 45 min intervals during the next 4.5 h. Each value is the mean ± s.e.m. of four to six observations.

Table 1 *In vitro* potency and duration of action of glycopyrrolate, tiotropium and ipratropium in reducing muscarinic-receptor mediated bronchoconstriction in human and guinea-pig airways

Compound	*Guinea-pig trachea*			*Human bronchus*		
	pIC_{50}[a]	$t_{1/2}$[b] (h)	*% recovery*[c] *(at 4.5 h)*	pIC_{50}	$t_{1/2}$ (h)	*% recovery (at 6.0 h)*
Tiotropium	9.1 ± 0.002	> 4	10 ± 4	9.5 ± 0.1	> 6	27 ± 3
Glycopyrrolate	9.0 ± 0.07	4.0 ± 0.5	50 ± 8	10.4 ± 0.02	3.7 ± 0.2	101 ± 10
Ipratropium	8.6 ± 0.02	0.5 ± 0.1	70 ± 7	9.5 ± 0.04	3.0 ± 0.2	110 ± 10

[a]pIC_{50} (or –log IC_{50}) represents the –log molar concentration of the test antagonist producing a 50% reversal of carbachol-induced tonic contraction in the guinea-pig isolated trachea or human isolated bronchus.
[b]$t_{1/2}$ (offset) is the time taken for response to carbachol to return to 50% recovery after washout of the test antagonist in the guinea-pig trachea (tiotropium, glycopyrrolate and ipratropium, 10 nM each) or human bronchus (tiotropium, 1 nM; glycopyrrolate, 3 nM and ipratropium, 10 nM).
[c]Percentage of response to carbachol obtained 4.5–6 h after the washout of the test antagonist. All data are mean ± s.e.m. of at least four observations.

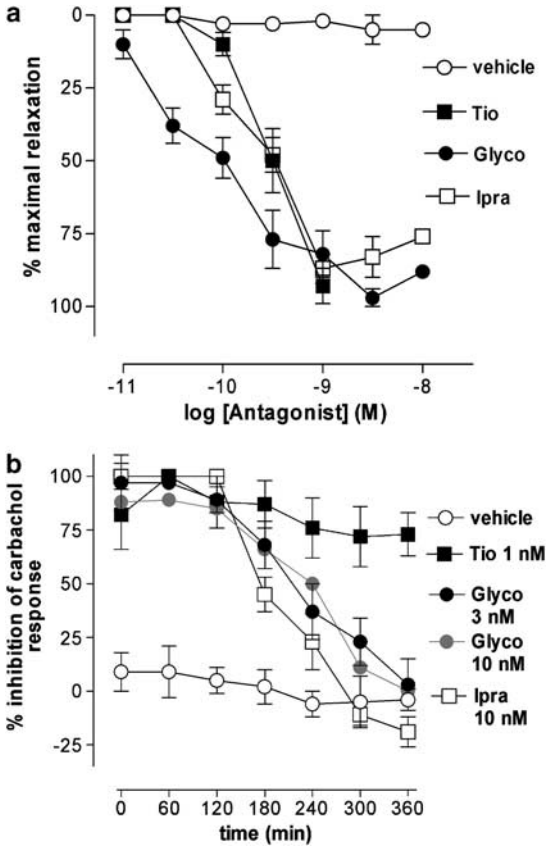

Figure 3 (a) Concentration-dependent inhibition by tiotropium (Tio), glycopyrrolate (Glyco) and ipratropium (Ipra) of carbachol (1 μM)-induced tonic contraction in the human isolated bronchus. (b) Decay of the inhibitory effects produced by the test compounds against contractile responses to carbachol (1 μM) in the human isolated bronchus. After washout of the antagonist (time = 0) carbachol was administered at 60 min intervals during the next 6.0 h. Each value is the mean ± s.e.m. of four to six observations.

Duration of antimuscarinic activity in the human isolated bronchus

As observed in the guinea-pig isolated trachea, carbachol (1 μM) produced highly reproducible tonic contractions over a period of several hours. Tiotropium, ipratropium and glycopyrrolate produced concentration-dependent inhibitions of carbachol (1 μM)-precontracted preparations (Figure 3a). Glycopyrrolate demonstrated to be a very potent inhibitor of carbachol-induced contractions ($pIC_{50} = 10.4$), being approximately 10-fold more potent than ipratropium ($pIC_{50} = 9.5$) or tiotropium ($pIC_{50} = 9.5$) (refer to Table 1). None of the three test compounds (10 nM each) inhibited a bronchial smooth muscle tone (averaging ~100% of that raised by carbachol) produced by neurokinin A (1 μM) ($n = 4$ each, data not shown).

A complete reversal of bronchial smooth muscle contraction was attained by all three antagonists (Figure 3a). The tiotropium (1 nM)-induced inhibitory effect persisted almost unaltered over a period of 6 h, irrespective of washout of the antagonist from the buffer solution (Table 1; Figure 3b). This is in agreement with literature that indicates that tiotropium undergoes a very slow dissociation from the human M_3 receptor (Disse *et al*., 1993). In contrast, ipratropium (10 nM)-induced inhibition proved to be rapidly reversible ($t_{1/2} \sim 3.0$ h), while the offset of glycopyrrolate-induced M_3 receptor blockade was intermediate between tiotropium and ipratropium. Indeed, a 50% reversal of glycopyrrolate (3 nM)-induced blockade occurred 3.7 h after washout of the antagonist (Table 1; Figure 3b). A higher concentration of glycopyrrolate (10 nM) failed to produce a longer inhibitory effect than that produced at 3 nM (Figure 3b).

Duration of antibronchoconstrictor activity in anaesthetized guinea-pigs

The effects of intratracheally instilled antimuscarinic compounds were assessed against ACh-induced bronchospasm in anaesthetized guinea-pigs. Both the maximum inhibition (peak effect) of ACh-induced bronchoconstriction and the inhibition still present 3 h and 24 h after the administration of each antagonist were evaluated. All the compounds exerted a dose-dependent prevention of ACh-induced bronchospasm, and produced a peak effect averaging ~80–90% inhibition, at the highest dose employed (Figure 4). Tiotropium was ~3-fold more potent than glycopyrrolate and ipratropium (cfr. ID_{50} values, Table 2). Tiotropium-induced inhibition lasted unchanged over a period of 3 h, and at the highest dose tested (1.3 nmol kg^{-1}) up to 24 h from antagonist administration (Figures 4a and 5; Table 2). Glycopyrrolate-induced inhibition remained quite stable up to 3 h, at all doses employed. At 24 h from administration, glycopyrrolate (1 nmol kg^{-1}) completely lost its inhibitory effect (not shown), whereas at 3 nmol kg^{-1} glycopyrrolate-induced effect was halved compared to that produced at 3 h (Table 2; Figures 4b and 5). The residual inhibitory effect of glycopyrrolate was also assessed at 16 h from administration, and proved to be as low as that found at 24 h (31.2 ± 10 vs 29.7 ± 6%, respectively) (Figure 5). Ipratropium-induced inhibitory activity was short lasting, as it declined quickly when compared to the other two antagonists. At 24 h from antagonist administration, ipratropium (1.45 nmol kg^{-1}) produced a negligible (14%) inhibition of ACh-induced bronchoconstriction (Table 2; Figures 4c and 5).

Discussion

In our study we addressed the question of the preclinical estimate of duration of action of glycopyrrolate in producing smooth muscle relaxation in the airways. To this end we have employed two isolated preparations: the guinea-pig trachea and the human bronchus. The ability of glycopyrrolate to sustain an inhibition of smooth muscle contraction triggered by muscarinic receptor activation in the airways was evaluated after the antagonist was washed out from the tissue. It is conceivable to speculate that the inhibitory effect of glycopyrrolate measured in our *in vitro* experiments may be due to a slow dissociation of the compound from muscarinic receptors. We have also estimated the duration of the antimuscarinic action of glycopyrrolate *in vivo*, as the ability of this compound to prevent ACh-induced bronchoconstriction in the guinea-pig. In this model, we presume that the inhibitory effect of glycopyrrolate results from both the rate of dissociation from muscarinic receptors and from additional pharmacokinetic factors including the retention of the compound in the lung, its metabolic stability and other factors. To our knowledge, this is the first *in vivo* animal model in which the duration

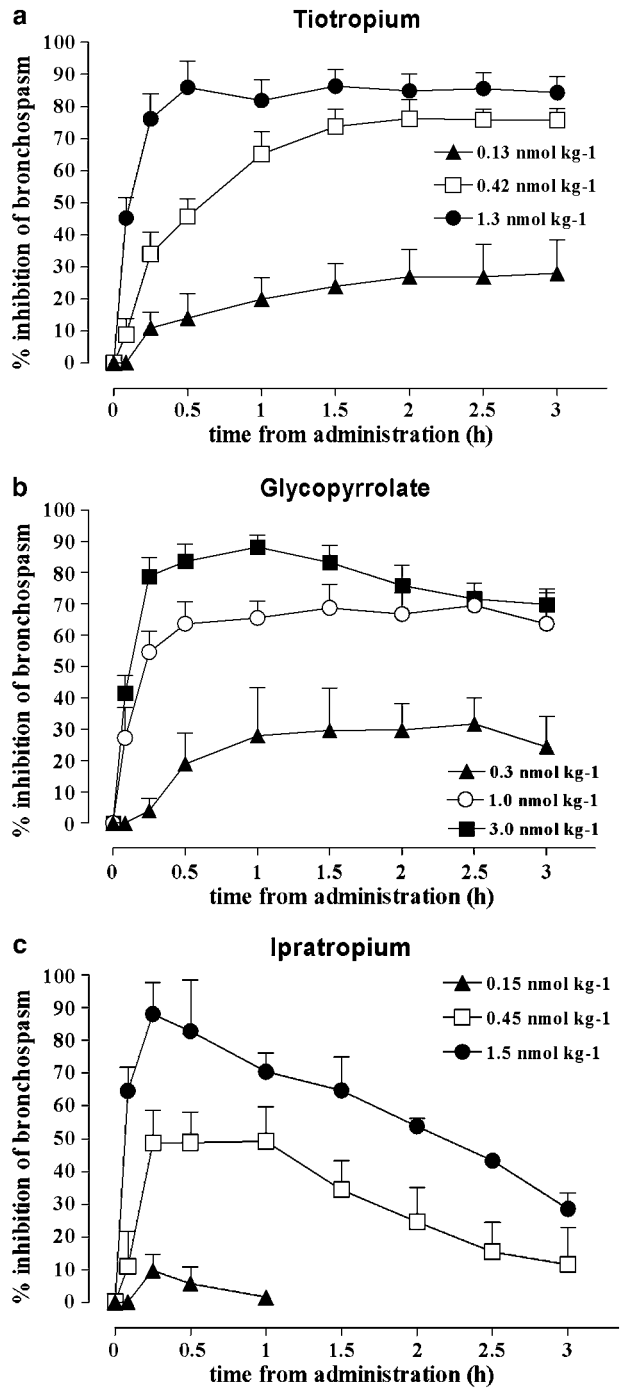

Figure 4 Inhibition afforded by intratracheally administered test compounds of ACh ($20\,\mu g\,kg^{-1}$ i.v.)-induced bronchoconstriction in anaesthetized guinea-pigs. ACh was injected before (baseline value) and up to 180 min after administration of the test compounds. Each value is mean ± s.e.m. of data obtained from four to seven animals.

of antimuscarinic action in the airways has been followed up to 24 h from antagonist administration. It should be underlined that in the sole study (Disse *et al*., 1993) reporting on tiotropium duration of action *in vivo*, the antibronchospastic effect of this drug was followed in the anaesthetized dog for 6 h only. As for glycopyrrolate, the information available about the duration of action of this drug is sparse *in vitro*, while it has never been assessed, in preclinical investigations, *in vivo*. Indeed, Haddad *et al*. (1999) have characterized the pharmacological profile of glycopyrrolate in human and guinea-pig airways, but in this study glycopyrrolate-induced smooth muscle inhibitory effect, assessed in guinea-pig isolated trachea and human isolated bronchus and trachea, was followed only up to 120 min from antagonist administration. This period of time may not be long enough to allow a comparison with tiotropium (whose inhibitory effect was reported to last for >300 min in human isolated airways; Takahashi *et al*., 1994).

Our present results show that glycopyrrolate duration of action is intermediate between that produced by tiotropium and that of ipratropium in both *in vitro* and *in vivo* bioassays. In our experiments, the enduring inhibitory effects of tiotropium lasted practically unchanged either in the isolated preparations or in the *in vivo* model. In this latter bioassay tiotropium-induced inhibition lasted up to 24 h after intratracheal administration of the drug. Our results with tiotropium are in agreement with both preclinical data (e.g. Takahashi *et al*., 1994) in which the effects of this drug were followed for shorter periods of time, and with its therapeutic profile. We have shown that glycopyrrolate-induced effects lasted longer as compared to that of ipratropium, but they were markedly shorter as compared to tiotropium. In particular, it should be noted that in the human bronchus glycopyrrolate-induced antimuscarinic effects disappear 6 h after the start of antagonist washout, whereas tiotropium still exerts ~2/3 of its initial inhibitory effect at the same time-interval. It is worth mentioning that our *in vitro* data with glycopyrrolate are partially in contrast with those reported by Haddad *et al*. (1999). In the former study, glycopyrrolate smooth muscle relaxing activity was maintained for a longer time in human bronchus than in guinea-pig trachea, where glycopyrrolate exerted a shorter inhibitory effect than ipratropium. These results were interpreted in terms of possible differences in the dissociation kinetics of glycopyrrolate and ipratropium from muscarinic receptors of different species, thus Haddad *et al*. (1999) suggested glycopyrrolate might have a therapeutic advantage over ipratropium as a bronchodilator in man. Indeed, in our study we found glycopyrrolate to exert more long-lasting effects than ipratropium in both guinea-pig and human airways. A less reproducible stimulus (i.e. electrical field stimulation) used by Haddad *et al*. (1999) compared to that used in our study (exogenous administration of carbachol) to evoke M_3 receptor-mediated contractions, and/or a shorter period of observation followed in the former study (100–120 min) compared to that adopted in our study (4.5–6 h) might be the cause of the discrepancy. As for the antagonist potencies of glycopyrrolate, tiotropium and ipratropium, the pIC_{50} (*in vitro*) or ID_{50} (*in vivo*) values for these compounds in our guinea-pig animal models were comparable. Although a contemporary evaluation of the three compounds has never been done before, our side-by-side evaluation is in accordance with similar data reported separately in other studies (e.g. Takahashi *et al*., 1994; Haddad *et al*., 1999). In the human bronchus, instead, glycopyrrolate was seven- to eight-fold more potent than tiotropium or ipratropium (present data). We do not have a simple explanation of this latter observation, which is not apparently supported by the binding affinities of the three test compounds at human M_3 receptor (Disse *et al*., 1993; Haddad *et al*., 1994, 1999). Rather, we think that the higher potency of glycopyrrolate might be due to the (faster) rate of onset of the smooth muscle relaxing activity of this

Table 2 *In vivo* potency and duration of action of glycopyrrolate, tiotropium and ipratropium in reducing muscarinic-receptor mediated bronchoconstriction in the anaesthetized guinea-pig

Compound	ID_{50}[a] (nmol kg^{-1})	*Dose* (nmol kg^{-1})	*Peak effect* (min)	*Peak effect*[b] (%)	*3* h *effect*[c] (%)	*24* h *effect*[d] (%)
Tiotropium	0.25 (0.09–0.7)	1.3	90	86.2±5.4	83.5±4.4^{NS}	70.6±6.0^{NS}
Glycopyrrolate	0.64 (0.2–1.9)	3	60	88.1±3.9	69.9±4.9*	29.7±6.2**
Ipratropium	0.49 (0.2–1.0)	1.45	15	88.1±9.6	28.3±4.8**	14.2±5.6**

[a]ID_{50} is the dose producing 50% inhibition of ACh-induced bronchoconstriction.
[b]The peak effect is the maximal inhibition produced by i.t. administration of each test compound.
[c]Inhibitory effect still present at 3 h after antagonist administration
[d]Inhibitory effect still present at 24 h after antagonist administration.
Results are presented as mean percent inhibition±s.e.m. of 4–7 animals group^{-1}. (*) Significantly different from peak effect: $P<0.05$ and (**) $P<0.01$. NS: not significant.

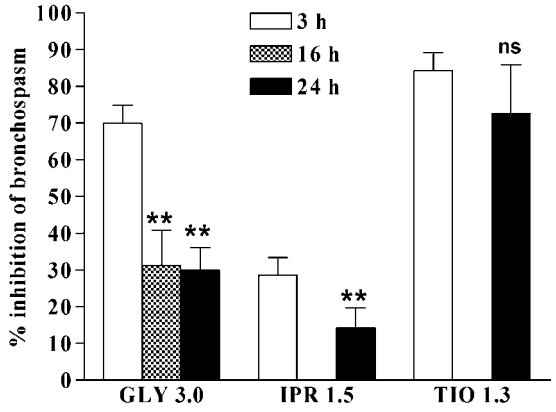

Figure 5 Comparison of the inhibitory effects produced by intratracheally administered test compounds (doses = nmol kg^{-1}) on ACh (20 μg kg^{-1} i.v.)-induced bronchoconstriction in anaesthetized guinea-pigs, at 3 vs 16–24 h from antagonist administration. (**) Significantly different from the value obtained at 3 h: $P<0.01$. Each value is mean±s.e.m. of data obtained from four to seven animals.

compound in the human bronchus, compared to the (slower) onset observed with the other two antagonists (data not shown). As a consequence, the potencies of tiotropium and ipratropium might be (slightly) underestimated in the human bioassay. The advantage of tiotropium over glycopyrrolate for the duration of action was confirmed in our *in vivo* test. Our data clearly show that 16–24 h after antagonist administration, glycopyrrolate-induced antimuscarinic effect is reduced to 1/3 of the initial response, whereas an equipotent dose of tiotropium is (almost) fully effective after the same interval (24 h) of time. This latter result, along with those obtained *in vitro*, suggest that a putative human therapy based on inhaled glycopyrrolate would probably be less effective as compared to tiotropium therapy, in terms of duration of action. However, a clinical report has recently been published (Hansel *et al*., 2005) showing that glycopyrrolate is able to produce very long-lasting effects in mild asthmatic patients. In this clinical study, Hansel *et al*. (2005) show that inhaled glycopyrrolate counteracts bronchoconstriction given by inhalation of methacholine, and that the protection lasts for 30 h. This prolonged effect of glycopyrrolate is achieved at doses (0.5–2.0 mg) that are 25–100 times higher than the effective dose of tiotropium (18 μg). The need to use such high doses was interpreted by Hansel *et al*. (2005) as evidence that glycopyrrolate is less potent than tiotropium at M_3 receptors. However, the explanation given by Hansel *et al*. (2005) is not supported by our results obtained in the human bronchus and guinea-pig trachea, showing that the potency of glycopyrrolate is greater than or equal to that of tiotropium, nor by previously published binding data (Disse *et al*., 1993; Haddad *et al*., 1994, 1999). We hypothesize that high doses of glycopyrrolate are needed not to compensate for its low potency, but to 'accumulate' this compound into the lungs and allow a long duration of action.

In conclusion, by following original procedures with classical *in vitro* and *in vivo* bioassays, we have obtained reproducible data on the duration of antibronchospastic activity of muscarinic M_3 receptor antagonists, up to 24 h from their administration. Glycopyrrolate, an antimuscarinic compound with bronchodilator activity potentially useful in asthmatic and COPD patients, has a longer duration of action than that of ipratropium in our guinea-pig or human bioassays, but is shorter acting at equipotent doses, than tiotropium. On this basis we speculate that a putative glycopyrrolate-based therapy for COPD might require more than once-a-day administration to be as effective as the currently employed once-daily therapy with tiotropium. The use of supramaximally effective doses of glycopyrrolate, however, might allow to overcome its shorter duration of action, but the question of possible greater side effects should be considered.

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