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Glycopyrronium bromide blocks differentially responses mediated by muscarinic receptor subtypes

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Summary. To analyse the potency of glycopyrronium bromide in blocking responses mediated via subtypes of muscarinic receptors in vitro, we tried to determine its equilibrium dissociation constants at prejunctional muscarinic receptors inhibiting the twitch response of rabbit vas deferens (presumed M_1 type), at M_2 (paced rat left atria), M_3 (guinea pig ileum) muscarinic receptor subtypes and at the muscarinic receptor of the rabbit iris sphincter (not M_1-M_4, not m_5). Glycopyrronium bromide shifted to the right the curve for inhibition of the twitch response induced by the agonist McN-A-343, and the methacholine-induced curves for inhibition of rat atrial contraction, and for tonic contraction of guinea pig ileum and rabbit iris sphincter.

Glycopyrronium bromide blocked with very high potency (>11, apparent $-\log K_B$) the response in rabbit vas deferens. Its affinity was low (9.09) for the M_2 subtype, and intermediate (10.31 or 10.13) for the ileal M_3 and the atypical iris muscarinic receptor subtype, respectively. Except at the receptors in rabbit vas deferens, the blockade of agonist effect appeared to be of simple competitive type.

In conclusion, glycopyrronium bromide is about 10 or 100 fold more potent in preventing a response to activation of the prejunctional receptor in rabbit vas deferens than in blocking an M_3 or M_2 muscarinic receptor subtype, respectively, in vitro. The low affinity for M_2 receptors may, in part, explain the low incidence of unwanted tachycardia in therapy. The drug failed to discriminate between an M_3 receptor and the atypical rabbit iris sphincter receptor.

Key words: Muscarinic receptor subtypes – Glycopyrronium bromide – Postjunctional dissociation constants – Rabbit iris sphincter

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Introduction

Glycopyrronium bromide (AHR 504, glycopyrrolate, 3-[α-cyclopentylmandeloyloxy]-1,1-dimethylpyrrolidinium bromide) is a potent quaternary antimuscarinic drug and widely used in anesthesia instead of other peripherally active muscarinic receptor antagonists (see Martindale 1989, Kentala et al. 1990). Since it was introduced into therapy long before subtypes of muscarinic receptors were known, surprisingly little is known about its selectivity for subtypes of muscarinic receptors in vitro. We therefore attempted to determine the equilibrium dissociation constants for glycopyrronium bromide in standard paradigms generally accepted to represent models for M_2, and M_3 subtypes (see Eglen and Whiting 1986; Hulme et al. 1990) and in two rabbit tissues, the vas deferens which is endowed with an inhibitory prejunctional receptor (suggested to be an M_1 subtype), and the iris sphincter which is endowed with an atypical muscarinic receptor (Bognar et al. 1992).

Materials and methods

Tissues were obtained from albino and non-albino rabbits (1.3–3.6 kg weight), guinea-pigs of either sex (150–300 g weight) and male albino rats (Wistar, 170–300 g weight) after killing by a sharp blow to the neck and exsanguination. All procedures on rabbit vas deferens, guinea pig ileum, and rabbit iris sphincter were carried out as described by Bognar et al. (1992) which are essentially similar to the following procedure used to obtain muscarinic receptor agonist concentration-response curves on cardiac M_2 receptors. Rat left atria were dissected and mounted between two parallel platinum electrodes in a jacketed organ bath (37 °C) and incubated with Tyrode solution (composition in mmol/l: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, D-glucose 5.6 and (+)-ascorbic acid 0.057) continuously bubbled with 5% CO_2 in O_2. The atria were stimulated electrically (2 Hz) at a current strength of 20% above threshold and were exposed twice to a single concentration of methacholine (10 μmol/l). Thereafter, 4 cumulative concentration-response curves to methacholine were carried out in each preparation. The second curve served as a control for the following curves which were carried in the absence or presence of

increasing concentrations of antagonist (50 or 90 min equilibration time; in case of 0.1 and 0.3 nmol/l extended to 5 and 3 h, respectively).

Results were expressed as arithmetic means with SE or geometric means with 95% confidence limits. The concentration of agonist at half-maximal effect was calculated by computer-assisted fitting of the experimental points to a sigmoidal curve. The ratio between individual EC_{50} values in the presence and absence of antagonist was used to calculate $-\log K_B$ values for the antagonists according to Arunlakshana and Schild (1959). In addition, $-\log K_B$ values were also estimated from individual dose ratios after constraining the slope to unity. Statistical differences between means were determined by use of Student's *t* test for paired or unpaired observations and, if more than one group of treatments was to be compared with a control group, by analysis of variance (ANOVA) followed by Dunnett's test.

The following drugs were used: glycopyrronium bromide (Brenner-Efeka, Münster, FRG); physostigmine salicylate (Merck, Darmstadt, FRG); (±)-methacholine chloride (Sigma, Deissenhofen, FRG); McN-A-343 ([4-hydroxy-2-butynyl]trimethylammonium chloride; RBI, Natick, MA, USA); yohimbine hydrochloride (Roth, Karlsruhe, FRG). Drugs were dissolved in distilled water.

Results

Control experiments

McN-A-343 inhibited the twitch response of the rabbit isolated vas deferens in a concentration-dependent and reversible manner. The twitch was abolished by the highest agonist concentration. In the absence of antagonist, up to five concentration-response curves could be obtained without changes in concentration at the half maximum effect or maximum response (see Bognar et al. 1992). Methacholine reversibly inhibited the contraction of rat isolated left atria. Whereas the maximum inhibition (e.g. 65±3.8%, $n = 3$, in the fourth curve) in time control experiments (no antagonist) was unchanged, a slight increase in sensitivity towards the agonist (decrease in IC_{50} compared to the 2nd curve) by 25–29% was observed. Experiments carried out in presence of antagonist were therefore corrected for the sensitivity shift in controls by adding to each individual dose ratio the corresponding mean time-induced shift observed in controls. Methacholine contracted the guinea pig ileum and the rabbit iris sphincter. In both tissues no time dependent changes in sensitivity or maximum effect were observed (not shown; see Bognar et al. 1992). The second curve always served as reference concentration-response curve in all tissues.

Effect of glycopyrronium bromide

The muscarinic receptor antagonist glycopyrronium bromide did not affect the basal twitch amplitude of the rabbit vas deferens, the basal contraction of rat left atria, or the basal tension of the guinea pig ileum and rabbit iris sphincter. The agonist concentration-response curves were shifted significantly by the antagonist to the right in a parallel manner without significant changes in maximum responses (for examples see Figs. 1–3).

The antagonism between McN-A-343 and glyocpyrronium bromide failed to follow the rules of simple competitive antagonism. The slope of the Schild regression line was lower than unity in the range of higher antagonist concentrations and steepened when the lowest concentration was also taken into account. A meaningful $-\log K_B$ value could therefore not be calculated as abscissa intercept. Assuming a slope of unity, from dose ratios of all antagonist concentrations a virtual apparent $-\log K_B$ value of 11.41±0.14 ($n = 22$) was estimated. At 0.3, 1, 3, and 10 nmol/l the mean of individual $-\log K_B$ values was 12.3, 11.7, 11.5, and 11.1 (each $n = 4-5$). At 0.1 nmol/l no shift ($n = 2$) was seen after an exposure time of 90 min. The shift seen after 3 h ($n = 4$, not shown) was still smaller than that after 5 h exposure time (see dose ratio in Fig. 4).

On rat atria glycopyrronium bromide shifted to the right the methacholine concentration-response curve without suppressing the maximum effect (e.g. inhibition in the fourth curve by 72±5.9% in presence of glycopyr-

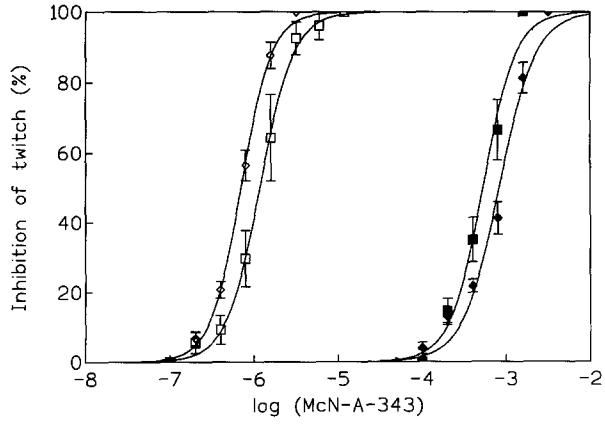

Fig. 1. Inhibition of field stimulation-evoked (0.05 Hz, 1 ms) twitch response of rabbit vas deferens incubated at 31 °C in Tyrode solution containing yohimbine 1 μmol/l. Inhibition (*ordinate scale*) is expressed as a percentage suppression of twitch. Symbols represent the mean (with SE as vertical lines) inhibition by McN-A-343 (*abscissa scale,* log molar concentration) from 5 corresponding experiments in the absence (*open symbols*) and in the presence of glycopyrronium bromide (■, 1 nmol/l, ◆ 10 nmol/l)

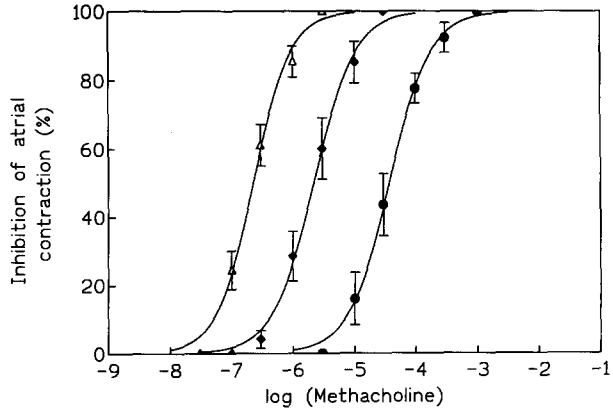

Fig. 2. Inhibition of field stimulation-evoked (2 Hz, 1 ms) contraction of rat isolated left atria (see Materials and methods). Inhibition (*ordinate scale*) is expressed as a percentage of maximum suppression which amounted to 61–72% reduction of isometric contraction (see also Results). Symbols represent the mean (with SE as vertical lines) inhibition by methacholine (*abscissa scale,* log molar concentration) from 5 experiments in the absence (*open symbols*) and in the presence of glycopyrronium bromide (◆, 10 nmol/l; ●, 100 nmol/l)

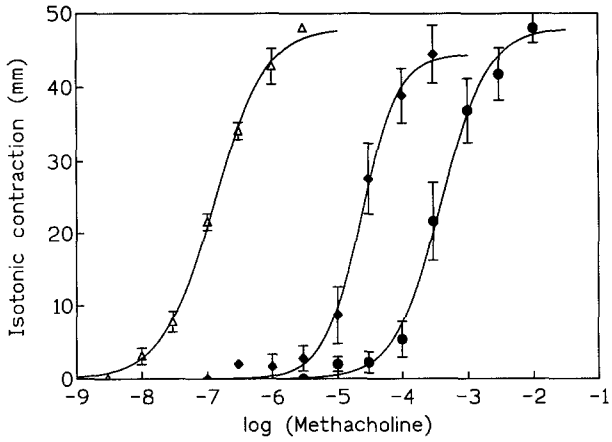

Fig. 3. Isotonic contraction of guinea pig isolated ileum (expressed in absolute shortening of individual preparations, mean with SE as vertical lines given in mm) induced by methacholine (*abscissa scale,* log molar concentration) from 5 experiments in the absence (*open symbols*) and in the presence of glycopyrronium bromide (◆, 10 nmol/l; ●, 100 nmol/l)

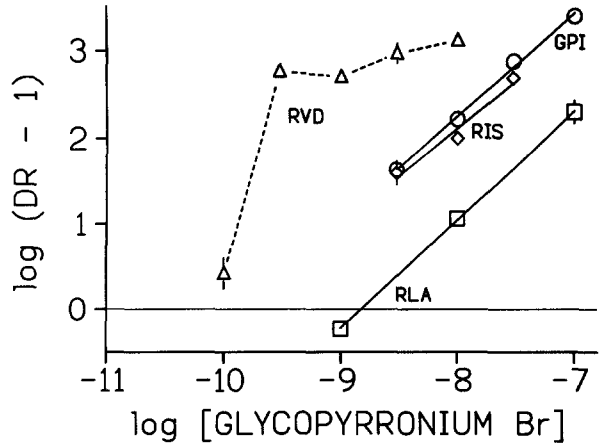

Fig. 4. The antagonism between glycopyrronium bromide and muscarinic agonists McN-A-343 (rabbit vas deferens, RVD) or methacholine (other preparations). The effects of the antagonists on the agonist curves were evaluated as the log of the dose ratio (log [DR−1], *ordinate*) according to the Arunlakshana-Schild procedure. Each point represents the mean with SE (within symbol size if not visible) of 4–5 (△, rabbit vas deferens), 3–6 (□, rat left atria, RLA), 4–5 (○, guinea pig ileum, GPI), and 4–5 (◇, rabbit iris sphincter, RIS) experiments. Results were obtained with different equilibration times to compensate for decreased equilibration time needed with increase of concentrations (5 h with 0.1 nmol/l, 3 h with 0.3 nmol/l, 90 min with 1–30 nmol/l, 50 min with 100 nmol/l). Straight lines were obtained by linear regression analysis of individual log (DR−1) determined at 50% response levels. For slopes see Table 1. *Dotted line* for rabbit vas deferens connects means obtained at each antagonist concentration and is not derived by linear regression

ronium bromide 100 nmol/l, for controls see above). The slope of the Schild plot was slightly larger than unity (1.27, Table 1). At a slope constrained to unity, an apparent $-\log K_B$ of 9.09 was estimated.

On guinea pig ileum and on rabbit iris sphincter glycopyrronium bromide shifted the methacholine-contraction curves to the right. Whereas on guinea pig ileum the maximum agonist effect was unchanged, 3 out of 5 iris preparations showed a decrease (by about 50%) of the maximum in the presence of the high (30 nmol/l) antagonist concentration. The slopes of Schild regressions tended to be larger than unity (1.16 and 1.08 on ileum and iris, respectively; Table 1, Fig. 4). The $-\log K_B$ values of 10.31 (guinea pig ileum, an M_3 subtype) and 10.13 (rabbit iris sphincter, atypical subtype) are indicative of an intermediate affinity of glycopyrronium bromide for these two receptor types, namely about 1/10 of the apparent $-\log K_B$ for receptors on rabbit vas deferens (presumably M_1), but 10 fold the affinity for rat atrial M_2 receptors.

Table 1. Comparison of $-\log K_B$ values for glycopyrronium bromide in various tissues presumably endowed with an M_1, M_2, M_3, and an atypical muscarinic receptor

	app. $-\log K_B$[a] (mean ± SE)	*n*	Slope of regression	$-\log K_B$ (95% conf. lim.)
Rabbit vas deferens (presumed M_1)	[>11[b]]	22	n.a.	n.a.
Rat left atria (M_2)	9.09 ± 0.08	14	1.27 ± 0.07	8.81 (8.69 – 9.01)
Guinea pig ileum (M_3)	10.31 ± 0.03	18	1.16 ± 0.04	9.98 (9.79 – 10.12)
Rabbit iris sphincter (atypical)	10.13 ± 0.06	14	1.08 ± 0.15	9.94 (9.5 – 10.79)

[a] Slope constrained to unity
[b] Minimum estimation, for details see Results
n.a., Not applicable

Discussion

Glycopyrronium bromide, a racemic mixture of a quaternary antimuscarinic drug, appears to have an interesting profile of selectivity for some subtypes of muscarinic receptors in vitro. A low concentration (1 nmol/l) of the drug shifted the concentration-response curve for McN-A-343 (prejunctional inhibition of twitch response) on the rabbit vas deferens to the right by a factor of about 500, and caused a shift of less than 2 fold of the methacholine concentration-response curve in rat left atria at identical equilibration times (Fig. 4). Furthermore glycopyrronium bromide 10 nmol/l shifted the agonist curve in the rabbit vas deferens to the right by nearly 1000 fold, but caused less than a 50 fold shift to the right of the agonist concentration-response curves in guinea pig ileum or rabbit iris sphincter (Fig. 4). A dissociation constant could not be calculated due to a lack of a linear proportionality between glycopyrronium bromide concentration and shift in agonist concentration-response curve in the rabbit vas deferens. The flat upper part of the dotted line formed by the points in Fig. 4 may in part result from non-muscarinic effects of McN-A-343. To reach maximum responses the agonist had to be applied in rather high concentrations (nearly 1000 fold its EC_{50}, Fig. 1). Testing a low antagonist concentration comparatively close to the apparent K_B resulted probably in incomplete drug equilibrium between organ bath and tissue biophase even after an equilibration time of 3 to 5 h and presumably caused the drop in agonist concentration ratio

(Fig. 4). The steep part of the dotted line in Fig. 4 may in part also be due to removal of low drug concentrations by an uptake system similar to the one which caused non-linearity (steepened slope at low antagonist concentrations) in Schild-plots for quaternary drugs in urinary bladder of the mouse (Durant et al. 1991). In the presence of all concentrations for which a more complete equilibrium was assumed, however, the preferential blockade exceeded a factor of 100 (rabbit vas deferens compared with rat atria) or 10 (rabbit vas deferens compared with guinea pig ileum or rabbit iris, respectively).

The reason for the selective blockade of the muscarinic response on rabbit vas deferens may be attributable to a selectivity for this inhibitory prejunctional muscarinic receptor (which is widely assumed to belong to the M_1 subtype). This appears unlikely to be the sole reason as we failed to observe clear competitive interaction between agonist and antagonist. An accumulation of antagonist in the surroundings of the receptor biophase could account for the observation. An indirect additional effect such as purinoceptor blockade, though appearing unlikely, cannot be excluded at present. A slope of less than unity may also be the result of the racemic nature of the compound, more than one stereoisomer being active at overlapping concentration ranges. Nevertheless glycopyrronium bromide appears to be one of the most potent antimuscarinics to block the prejunctional muscarinic response of rabbit vas deferens (see Eltze 1988; Eltze and Figala 1988; see review of papers in Dörje et al. 1991).

The observation that the M_3 receptor (on guinea pig ileum) was preferentially blocked by glycopyrronium bromide as compared to the M_2 subtype (in rat atria) was not totally unexpected in view of clinical observations (see below). The degree of selectivity approaches that of other drugs with an element of M_3-selectivity such as 4-DAMP (Barlow et al. 1976) or hexahydrosiladifenidol (Fuder et al. 1985). Interestingly glycopyrronium bromide is some ten fold more potent in blocking M_3 receptors than atropine which has $-\log K_B$ values of around 9.3 (see Hulme et al. 1990). Thus it is one of the most potent M_3 subtype blockers used in current therapy or experimental pharmacology (see Doods 1991). At the M_2 receptor, glycopyrronium bromide is at best equipotent with atropine as reflected by virtually identical $-\log K_B$ values. The M_3-selectivity could have implications for unwanted drug effects. At low concentrations the drug would be expected to block glandular secretion without causing tachycardia. Indeed in a study (Dworacek et al. 1987), in which the reversal of neuromuscular blockade by neostigmine was investigated in humans, glycopyrronium bromide caused less tachycardia than methylatropine bromide, another peripherally active antimuscarinic drug (for a review of earlier similar observations see Martindale 1989). Interestingly, low doses of glycopyrronium bromide were reported to induce a small decrease in heart rate in volunteers (Mirakhur 1979). Such "paradoxical", "parasympathomimetic" cardiac effects (i.e. bradycardia) were not always seen in premedicated patients or volunteers and sometimes less frequent with glycopyrronium bromide than with atropine (Berger et al. 1988; Ali-Melkkilä et al. 1991). Glycopyrronium bromide was used as a long acting bronchodilator (Slovis et al. 1987; Walker et al. 1987; Schroeckenstein et al. 1988; Gilman et al. 1990). Bronchoconstriction is thought to be mediated by M_3 receptors (Eglen and Whiting 1988).

Glycopyrronium bromide failed to discriminate between the M_3 and the atypical iris sphincter receptor. Initially we had decided to investigate this quaternary drug (with remote structural similarity to valethamate bromide), because another quaternary drug, valethamate bromide, had in our hands a high and equal affinity for the M_1-M_3 subtypes, but was 1/10 as potent at the iris receptor (Bognar et al. 1992) thus contributing to a wealth of data indicating differences between the iris muscarinic receptor (Fuder et al. 1989; Bognar et al. 1992) and functionally characterized M_1-M_4 or M_5 receptors (see Hulme et al. 1990). This discriminating property was expressed even more in the case of clozapine (Bognar et al. 1992). In this respect glycopyrronium bromide only partly met our expectations: the data presented here confirm at least that the iris receptor is not an M_2 type or similar to the prejunctional receptor of the rabbit vas deferens.

Since glycopyrronium bromide contains two asymmetrical carbon atoms and the drug may consist of two pairs of diastereomers, the dissociation constants given in this paper may represent underestimations of true constants of the most active stereoisomer(s) by a factor of up to four. The subtype selectivity of the single enantiomers cannot be determined at present, since isolated isomers are not available.

In conclusion, the results with glycopyrronium bromide presented here reveal an interesting profile of selectivity for subtypes of muscarinic receptors which may, in part, provide an explanation for clinical observations with regard to wanted and unwanted antimuscarinic effects. In addition to special pharmacokinetic properties (see Kanto and Klotz 1988; Ali-Melkkilä et al. 1989), the subtype selectivity may confer a useful activity profile to this mixture of diastereomers of glycopyrronium bromide. An even more interesting aspect would be a differential subtype selectivity profile of individual stereoisomers. This aspect should stimulate efforts to separate the isomers.

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