Reversible antagonism by anticholinergic drugs of acetylcholine binding to muscarinic receptors leads to a wide range of peripheral and central effects [1, 2]. Molecular cloning studies have shown that muscarinic receptors comprise a family of five related genes, designated m₁–m₅ [3–6], although only four different pharmacologically identifiable subtypes of muscarinic receptor have been proposed: M₁ (neuronal tissue), M₂ (heart tissue), M₃ (exocrine glands) and M₄ (NG108-15 cells and rabbit lung). This classification is currently defined on the basis of actions in functional and binding experiments of several selective antagonists such as pirenzepine for the M₁ subtype, otenzepad for the M₂ subtype and hexahydrosiladiphenidol (HHSiD) for the M₃ subtype. The rank order of competition of these ligands has been established for the M₁ receptor as: atropine > 4-diphenylacetoxy-N-methylpiperidine(4-DAMP) > otenzepad > HHSiD > pirenzepine, and for the M₂ receptor as: atropine > 4-DAMP > HHSiD > pirenzepine > otenzepad [7–10].

Block of M₂ and M₃ subtypes appears to mediate increased heart frequency (M₂ block) and inhibition of salivation and bronchial secretion (M₃ block) produced by the antimuscarinic drugs atropine and glycopyrronium [11, 12]. Both antimuscarinic drugs are used widely as perioperative medication to prevent cardiac arrhythmia and bradycardia [13–16], and to inhibit salivation and excessive respiratory tract secretions [17–21]. When glycopyrronium and atropine antiasialagogic activity are comparable, glycopyrronium is thought to cause less tachycardia while simultaneously blocking bradycardia more effectively [22–26] than atropine. This suggests that glycopyrronium may show different affinities for the M₂ and M₃ muscarinic receptor subtypes. The purpose of this study was therefore to use receptor binding methods to analyse the affinity and selectivity of glycopyrronium for M₂ and M₃ muscarinic receptor subtypes. The materials and methods of this study was therefore to use receptor binding methods to analyse the affinity and selectivity of glycopyrronium for M₂ and M₃ muscarinic receptor subtypes obtained from rat ventricle and submandibular gland, respectively. These results were compared with the findings obtained with atropine, a classic non-selective antimuscarinic compound, and the selective antimuscarinic drugs pirenzepine as an M₁ antagonist, otenzepad as an M₂ selective muscarinic antagonist, and the M₃ selective antimuscarinic drug HHSiD.

Materials and methods

Experimental animals were handled throughout in accordance with the Guiding Principles in the Care and Use of Animals, as approved by the American Physiological Society. After institutional approval by our Animal Care Committee, 55 male Wistar rats, weighing 250–300 g (Biocenter, Barcelona), were
killed by cervical dislocation. The ventricles and both submandibular glands were removed. The tissues were cleaned and homogenized at 1:250 (w/v) (ventricle) or at 1:150 (w/v) (submandibular gland) with a mixer (Ultra-Turrax) at the 70 % power setting for 30 s in Na\textsuperscript{+}/Mg\textsuperscript{2+} HEPES buffer, pH 7.4 (NaCl 100 mmol litre\textsuperscript{-1}, MgCl\textsubscript{2} 10 mmol litre\textsuperscript{-1}, HEPES 20 mmol litre\textsuperscript{-1}). The homogenates were filtered through four layers of buffer-soaked cheesecloth. The protein concentration of the crude homogenate was determined by the method of Lowry and colleagues [27]. The final protein concentration was adjusted with buffer to 2 mg ml\textsuperscript{-1} (ventricle) or 2.5 mg ml\textsuperscript{-1} (submandibular gland).

All binding assays were performed in triplicate with \[^3H\]-N-methyloscopalamine (\[^3H\]-NMS) as the radioligand. Incubation volumes for all bindings assays were 1 ml. The reaction was started by adding tissue homogenate, and was allowed to proceed for 45 min at 30 °C in a water bath [28-30]. Preliminary kinetic experiments indicated that this incubation time was sufficient to reach equilibrium at the \[^3H\]-NMS concentration used in these studies. The reaction was stopped by adding 4 ml of ice-cold Tris-HCl buffer 20 mmol litre\textsuperscript{-1} pH 7.4. The contents of the tubes were filtered immediately through Whatman 24-mm GF/B filters under a vacuum in a Brandel Cell Harvester Filtration unit. The filters were washed twice with 4 ml of ice-cold Tris-HCl buffer. Non-specific binding was determined by parallel triplicate assays containing atropine sulphate 1.0 \mu mol litre\textsuperscript{-1}. This concentration was found to define non-specific binding in previous studies. Liquid scintillation counting of the filters was performed in 4 ml of Optiphase “Hisafe” 3 (LKB Wallac) using a LKB beta spectrometer with a counting efficiency of 60%.

In saturation binding experiments, eight concentrations of \[^3H\]-NMS (from 0.01 to 6 nmol litre\textsuperscript{-1}) were incubated with the membranes at equilibrium. In competition binding experiments, a single concentration of the radioligand (0.4-0.6 nmol litre\textsuperscript{-1}) was incubated with the membranes in the presence or absence of competitors. At least 15 concentrations (ranging from 1000 times lower to 1000 times greater than the IC\textsubscript{50} value for the compound studied) were used for each compound studied.

All binding data were analysed with the iterative curve-fitting computer package Radlig [31], which includes the program Ligand [32]. In competition binding experiments the IC\textsubscript{50} values were corrected to their respective K\textsubscript{i} values using the Cheng–Prusoff approximation. All results are mean (SEM) of four to six experiments.

\[^3H\]-NMS (specific activity 84 Ci mmol\textsuperscript{-1}) was obtained from Amersham (Amersham, London, UK). Atropine sulphate and all other chemicals and reagents were purchased from the Sigma Chemical Co. (St Louis, MO, USA). Hexahydrodiphenidol hydrochloride (HHSID or cyclohexylphenyl[3-piperidinopropyl]silanol hydrochloride) was obtained from Research Biochemicals Incorporated (RBI, UK). Pirenzepine hydrochloride (5,11-dihydro-11-(4-methylpiperazin-1-ylacetlyl)pyridol[2,3-b][1,4]-benzodiazepin-6-one dihydrochloride monohydrate) was generously provided by Boehringer Ingelheim (Ingelheim, Germany). Otenzepad (AF-DX 116 or 11-[(2-diethylamino)ethyl]-1-piperidinyl]-acetyl-5,11-dihydro-6H-pyrido [2,3-b][1,4]-benzodiazepine-6-one) was generously provided by Dr K. Thomae Gmbh (Biberach, Germany), and glycopyrronium (3-(alpha-cyclopentylmandeloloyloxy)-1,1-dimethylpyrrolidinium bromide) was generously provided by Wyeth-Ayerst (Princeton, NJ, USA).

**Results**

\[^3H\]-NMS bound specifically and saturably to an apparently homogeneous population of non-interacting binding sites in membrane homogenates from the rat ventricle and submandibular gland. Within the concentration range 0.01-6 nmol litre\textsuperscript{-1}, the specific binding of \[^3H\]-NMS had similar dissociation constants (K\textsubscript{d} values) in the ventricle (K\textsubscript{d} = 0.834 (0.154) nmol litre\textsuperscript{-1}) and submandibular gland (K\textsubscript{d} = 1.81 (0.33) nmol litre\textsuperscript{-1}), with a Hill coefficient close to unity in both tissues. Maximum binding (Bmax) was 62.55 (12.97) fmol/mg of protein for rat ventricle and 15.32 (2.59) fmol/mg of protein for rat submandibular gland. Non-specific binding was 15% for the ventricle (non-specific binding at the radioligand K\textsubscript{d} was 8%) and 40% for the submandibular gland (non-specific binding at the radioligand K\textsubscript{d} was 23%) or less of the total binding.

Figures 1 and 2 show the displacement curves of pirenzepine, otenzepad, HHSID, glycopyrronium and atropine in the ventricle and submandibular gland, respectively. The inhibition constants (K\textsubscript{i}) values) and slope values (Hill coefficients) of the antagonists against \[^3H\]-NMS in both tissues are shown in table 1. The order of inhibition of \[^3H\]-NMS binding by the unlabelled drugs differed in the two tissues and were: glycopyrronium > atropine > otenzepad > HHSID > pirenzepine in the rat ventricle, and glycopyrronium > atropine > HHSID > pirenzepine > otenzepad in the rat submandibular gland. The inhibition concentration curves produced by all antagonists were steep and parallel, and the
Muscarnic receptor subtypes. In agreement with possible selectivity of glycopyrronium for M2 and M3 tissue, showing similar affinities (P > 0.05) binding by unlabelled compounds in rat submandibular gland homogenates. Data illustrate a representative experiment in triplicate. □ = Atropine; ■ = glycopyrronium; △ = pirenzepine; ● = otenzepad; ○ = HHSID.  

Table 1 Binding variables (mean ± SEM) derived from competition experiments (n = 4–6 experiments). Inhibition curves were obtained in the presence of [3H]-NMS methyskopoline 0.4–0.6 nmol litre⁻¹. Significant differences (P < 0.01) compared with: ** submandibular gland; †† ventricle; ††† atropine (Student’s t test)  

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ventricle M2 subtype</th>
<th>Submandibular gland M3 subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>4.29 (0.21)/</td>
<td>4.85 (1.65)/</td>
</tr>
<tr>
<td></td>
<td>1.02 (0.02)</td>
<td>0.98 (0.03)</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>1224.7 (142.61)/</td>
<td>1102.4 (112.27)/</td>
</tr>
<tr>
<td></td>
<td>0.59 (0.01)</td>
<td>0.97 (0.03)</td>
</tr>
<tr>
<td>Otenzepad</td>
<td>235.92 (36.189)**/</td>
<td>10478 (1121.0)/</td>
</tr>
<tr>
<td></td>
<td>1.00 (0.02)</td>
<td>1.02 (0.02)</td>
</tr>
<tr>
<td>HHSID</td>
<td>272.54 (23.390)/</td>
<td>38.28 (1.69)††</td>
</tr>
<tr>
<td></td>
<td>0.97 (0.01)</td>
<td>1.00 (0.02)</td>
</tr>
<tr>
<td>Glycopyrronium</td>
<td>1.88 (0.04)‡‡</td>
<td>1.68 (0.18)‡‡</td>
</tr>
<tr>
<td></td>
<td>0.98 (0.01)</td>
<td>0.98 (0.01)</td>
</tr>
</tbody>
</table>

slopes, or Hill coefficients, of the Hill plots were close to unity for both the ventricle and submandibular gland, which indicated that the unlabelled antagonist and [3H]-NMS, were bound to a homogeneous population of binding sites. Atropine, glycopyrronium and pirenzepine did not distinguish between the M2 (ventricle) and the M3 (submandibular gland) sites. Otenzepad and HHSID showed at least 10-fold selectivity between the sites in the ventricle and submandibular gland (P < 0.01). Otenzepad binding was weaker at M3 sites (submandibular gland), and HHSID binding was weaker at M2 sites (ventricle). In common with atropine and pirenzepine, glycopyrronium bound to a homogeneous population of muscarinic receptors in both ventricle (Kd = 1.8892 (0.0496) nmol litre⁻¹) and submandibular gland (Kd = 1.6867 (0.1848) nmol litre⁻¹) tissue, showing similar affinities (P > 0.05) for M2 and M3 muscarinic receptor subtypes.

Discussion

The aim of the present study was to determine the possible selectivity of glycopyrronium for M2 and M3 muscarinic receptor subtypes. In agreement with studies in other rat tissues, [3H]-NMS binding to rat ventricle and submandibular gland homogenates appeared to comprise a single population of binding sites. In addition, the Hill coefficients were close to unity. Affinity (Kd) and receptor density (Bmax) obtained in the rat ventricle and submandibular gland agreed with those reported previously [29, 33]. In competition experiments, all compounds competitively inhibited the binding of the radioligand to the binding sites in both the ventricle and submandibular gland homogenates. The affinities (Kd) values in both tissues were consistent with those of muscarinic receptors (table 1). The rank orders of antimuscarinic displacement against [3H]-NMS obtained in this study were in agreement with those given in earlier studies [8, 9]. In the rat ventricle, the rank order of affinity was: glycopyrronium > atropine > otenzepad > HHSID > pirenzepine, which was similar to that reported for the M3 subtype. In the rat submandibular gland, the rank order of affinity was: glycopyrronium > atropine > HHSID > pirenzepine > otenzepad, which was similar to that found for the M3 subtype. As with atropine, competition binding experiments with glycopyrronium generated curves displaying Hill coefficients close to unity in both tissues (table 1), indicating the presence of a single population of non-interacting binding sites in both the ventricle and submandibular gland. Glycopyrronium showed similar affinity (Kd value) for both the M3 (rat ventricle homogenates) (Kd = 1.8892 (0.0496) nmol litre⁻¹) and the M3 muscarinic receptor subtype (rat submandibular gland) (Kd = 1.6868 (0.1848) nmol litre⁻¹). Glycopyrronium showed higher affinity than atropine (P < 0.01) for M3 and M4 muscarinic receptor subtypes in rat tissue homogenates (table 1).

The absence of selective binding of glycopyrronium to M2 and M3 muscarinic receptor subtypes in this study does not support a pharmacodynamic basis for the specific trophism or greater effect of this compound in preventing cardiac arrhythmias described elsewhere. However, radioligand binding experiments do not take into account all pharmacodynamic characteristics elicited by a compound and cannot predict “in vivo” selectively. In isolated organ experiments with rat atria and guineapig ileum, glycopyrronium showed lower affinity (apparent – log KB = 9.09) for the rat left atrium (M3 subtype) and higher affinity (apparent – log KB = 10.31) for the guineapig ileum (M3 subtype), indicating that glycopyrronium may discriminate between the two subtypes in these experimental assays [34]. These data are compatible with a previous description of several peculiar characteristics of glycopyrronium with respect to other anticholinergic drugs. For example, allosteric interaction may occur between this compound and binding sites present in cardiac membranes [35], and glycopyrronium had the opposite effect with respect to atropine in rat phrenic nerve-diaphragm preparation, where glycopyrronium depressed neuromuscular transmission whereas atropine enhanced this process [36].

We conclude that glycopyrronium has no selectivity for either M2 or M3 muscarinic receptor.
subtypes in radioligand binding experiments, and that it is not possible to explain the different pharmacological effects of glycopyrronium and atropine on the basis of their hypothetical selectivity patterns for muscarinic receptor subtypes.

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References