Neuromuscular Relaxants as Antagonists for $M_2$ and $M_3$ Muscarinic Receptors

Vivian Y. Hou, M.D.,* Carol A. Hirshman, M.D.,† Charles W. Emala, M.D.‡

Background: Neuromuscular relaxants such as pancuronium bind to $M_3$ and $M_4$ muscarinic receptors as antagonists. Blockade of muscarinic receptors in atria of the $M_2$ subtype mediates tachycardia. In the lung, blockade of $M_2$ receptors on parasympathetic nerves potentiates vagally induced bronchospasm, whereas blockade of $M_3$ receptors on bronchial smooth muscle inhibits bronchospasm. The current study was designed to quantify the affinity of a series of neuromuscular relaxants for the $M_2$ and $M_3$ muscarinic receptors, which were individually stably transfected in Chinese hamster ovary cell lines.

Methods: Competitive radioligand binding assays determined the relative binding affinities of the neuromuscular relaxants pancuronium, succinylcholine, mivacurium, doxacurium, atracurium, rocuronium, gallamine, and pipercuronium for the muscarinic receptor in the presence of a muscarinic receptor antagonist (H-QNB) in membranes prepared from cells individually expressing either the $M_2$ or $M_3$ muscarinic receptor.

Results: All muscle relaxants evaluated displaced $^3$H-QNB from muscarinic receptors. The relative order of potency for the $M_2$ muscarinic receptor (highest to lowest) was pancuronium, gallamine, rocuronium, atracurium, pipecuronium, doxacurium, mivacurium, and succinylcholine. The relative order of potency for the $M_3$ muscarinic receptor (highest to lowest) was pancuronium, atracurium, pipecuronium, rocuronium, mivacurium, gallamine, succinylcholine, and doxacurium.

Conclusions: All neuromuscular relaxants studied had affinities for the $M_2$ and $M_3$ muscarinic receptor, but only pancuronium and gallamine had affinities within the range of concentrations achieved with clinical use. The high affinities of gallamine and pancuronium for the $M_3$ muscarinic receptor are consistent with a mechanism of $M_3$ receptor blockade in relaxant-induced tachycardia. (Key words: Chinese hamster ovary cells; radioligand binding; stable transfection; tachycardia.)

MUSCARINIC cholinergic receptors found on postganglionic neurons, cardiac muscle, smooth muscle, and glands mediate important physiologic functions such as heart rate, airway caliber, and salivation. Molecular cloning studies have identified five different subtypes of muscarinic receptors encoded by five distinct genes ($m_1$, $m_2$, $m_3$, $m_4$, and $m_5$), although pharmacologic techniques only distinguish between the $M_1$, $M_2$, $M_3$, and $M_4$ muscarinic receptors.¹

Neuromuscular blockers commonly used in anesthetic practice interact with muscarinic and nicotinic receptors.²,³ Agents that are antagonists for the $M_2$ muscarinic receptor should increase heart rate because the heart expresses a relatively pure population of $M_2$ receptors.⁴ Tachycardia results from blockade of $M_3$ muscarinic receptors on both cardiac myocytes and vagal efferent fibers. Similarly, $M_2$ selective muscarinic antagonists should potentiate vagally induced bronchoconstriction,⁵,⁶ because $M_2$ receptor activation also inhibits the release of acetylcholine from postganglionic parasympathetic fibers in the airway. Conversely, neuromuscular blockers that selectively inhibit the $M_1$ muscarinic receptor on the smooth muscle in the airway should inhibit vagally induced bronchoconstriction, because $M_1$ receptor activation mediates smooth muscle contraction in the airway.⁶ Therefore, $M_2$ selective muscarinic antagonist activity that occurs at doses of neuromuscular relaxants in the clinical range is undesirable because of the side effects of tachycardia and bronchospasm, whereas $M_1$ selective muscarinic receptor antagonist activity that occurs in the dose of the neuromuscular relaxant used clinically is desirable as it results in decreases in airway tone and salivary secretions. Gallamine, a neuromuscular blocker no longer used because

---

¹ Fellow, Department of Anesthesiology and Critical Care Medicine.
² Professor, Departments of Anesthesiology, Environmental Health Sciences, and Medicine.
³ Associate Professor, Department of Anesthesiology and Critical Care Medicine.

Received from the Departments of Anesthesiology and Critical Care Medicine, and Environmental Health Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland. Submitted for publication February 12, 1997. Accepted for publication October 28, 1997.

Address reprint requests to Dr. Emala. The Johns Hopkins School of Hygiene & Public Health, Division of Physiology/Room 7006, 615 North Wolfe Street, Baltimore, Maryland 21205. Address electronic mail to: cemala@welchlink.welch.jhu.edu

Anesthesiology, V 88, No 3, Mar 1998
of a high prevalence of undesirable side effects, was later discovered to be a highly selective M₂ muscarinic receptor antagonist.¹

Because lung and most other tissues express a mixed population of muscarinic receptors, and because the differences in affinity for even subtype-selective muscarinic antagonists are often minimal, the estimation of the affinity of the neuromuscular blockers for M₂ and M₁ receptors cannot be calculated with any degree of precision in tissues coexpressing multiple muscarinic receptor subtypes. Pure populations of muscarinic receptors in a tissue provide a unique way of obtaining pharmacologic profiles of each receptor subtype. Until recently, heart tissue was thought to express a pure population of M₂ muscarinic receptors, but molecular and functional evidence exists for the expression of M₁ muscarinic receptors also.⁷ Many peripheral tissues, such as smooth muscle of airway and gut, that express M₁ muscarinic receptors also express M₂ muscarinic receptors; therefore, we used two separate cell lines, one expressing the M₂ and the other expressing the M₁ muscarinic receptor, to characterize a series of commonly used neuromuscular blocking drugs regarding their affinities at the M₂ and M₁ muscarinic receptor.

Materials and Methods

Drugs and Reagents

Pipercuronium and rocuronium were gifts from Organon (West Orange, NJ); doxacurium, mivacurium, and atracurium were gifts from Glaxo-Wellcome (Research Triangle Park, NC). Pancuronium and gallamine were purchased from Sigma (St. Louis, MO). The muscarinic receptor antagonist [³⁵S]QNB was purchased from Amersham Life Science (Arlington Heights, IL). All drugs were dissolved in dH₂O. Chinese hamster ovary (CHO) cells stably transfected with complementary deoxyribonucleic acid encoding the rat M₃ muscarinic receptor were purchased from the American Type Culture Collection (Rockville, MD). Chinese hamster ovary cells stably transfected with complementary deoxyribonucleic acid encoding the human M₂ muscarinic receptor were provided by Norman Lee, Ph.D. (The Institute for Genome Research, Rockville, MD).

Cell Culture

Chinese hamster ovary cells stably transfected with either the M₂ or M₃ muscarinic receptor were grown in Dulbecco’s modified essential medium (DMEM) media containing 10% fetal bovine serum and antibiotic agents (100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B, and 100 U/ml nystatin). Cells were cultured in T500 flasks (500 cm²) at 37°C in a humidified atmosphere of 5% CO₂/95% air and harvested at confluence.

Preparation of Chinese Hamster Ovary Cell Membranes

At confluence, culture media were removed from flasks. Cells were incubated in lysis buffer (10 mm HEPES, 2 mm ethylenediaminetraacetic acid, 100 µm PMSF, pH 8.0) at 37°C in a carbon dioxide incubator until detached (20–40 min). Lysed cells were centrifuged at 48,000 × g (Sorvall RC-5B with SS-34 rotor, Newton, CT) for 20 min at 4°C. Cold HEPES buffer (100 mm, pH 7.4) was used to resuspend the pellet after the removal of the supernatant. The lysates were washed two additional times, and the final pellet was resuspended in 6 ml of HEPES buffer at 2–5 mg/ml and stored at −70°C until used for radioligand binding assays.

Saturation Radioligand Binding

Forty micrograms of CHO cell membrane proteins was incubated with increasing concentrations of the radioligand [³⁵S]QNB (47 Ci/mmol; 0.05–3.00 nm) in the presence or absence of atropine (2 µm) in binding buffer (40 mm KH₂PO₄, 160 mm K₂HPO₄, in 50 mm NaCl, pH 7.4). All radioligand experiments were incubated for 2 h at room temperature in a final volume of 1 ml. Preliminary experiments confirmed that the 2-h incubation period was adequate to achieve equilibrium binding. All binding experiments were terminated by filtration through GF/B glass fiber filters and washed three times with 5 ml of cold 0.9% NaCl using a cell harvester (Brandell, Gaithersburg, MD). Filters were immersed in 5 ml of Econo scintillation fluid, stored overnight, and counted in a scintillation counter (Beckman LS 5000 TD; Beckman, Fullerton, CA) with an efficiency of 45–50%. Specifically bound counts were analyzed by linear regression after Scatchard transformation using the EBDA computer program⁸ to obtain the line of best fit.

Competitive Radioligand Binding Assays

Forty micrograms of CHO cell membrane protein was incubated with [³⁵S]QNB (0.18 nm) and muscle relaxants of increasing concentrations (10⁻¹⁰–10⁻³ m), under con-
ditions described previously for saturation experiments. The chosen radioligand concentration (0.18 nM) for the competition experiments was 3.6 times the equilibrium constant (Kd) of the M2 muscarinic receptor and 1 Kd of the M1 muscarinic receptor. The competitive displacement of [3H]QNB by increasing concentrations of muscle relaxants was analyzed by nonlinear regression. A reiterative curve-fitting program, Inplot 4.0 (Graph Pad, San Diego, CA) was used to calculate the relative binding affinity (IC50) values using a four-parameter logistic equation (log scale) with the slope factor set to -1.

**Statistical Analysis**

The Kd of [3H]QNB and the receptor numbers of the M2 or M1 muscarinic receptor in CHO cell membranes were calculated from radioligand saturation binding assays after Scatchard transformation. The Kd was determined from the negative reciprocal of the slope of the line and the maximum number of binding sites (Bmax), were determined from the x-intercept. IC50 values were obtained by nonlinear regression analysis of competitive radioligand displacement curves using the values from the reiterative curve-fitting program Inplot using a four-parameter logistic equation (log scale) with the slope factor set to -1.

**Results**

All data are as mean ± SE unless otherwise indicated. The CHO M2 and CHO M1 cells were first characterized for the level of expression of muscarinic receptors and for receptor affinity for the antagonist [3H]QNB and the agonist carbachol. Saturation of specific binding was achieved in the membranes of the CHO M2 or CHO M1 cells over the range of [3H]QNB used (0.01-3.00 nM; n = 3; figs. 1A and 1B). Hill coefficients were 1.03 ± 0.14 and 0.92 ± 0.04 for M2 and M1 muscarinic receptors, respectively, which was indicative of a single class of binding sites in both CHO M2 and CHO M1 cells (n = 3). Scatchard transformation of saturation binding showed that the CHO M2 membranes contained 681 ± 7 fmol/mg protein of receptor (Bmax) with an affinity (−log Kd) of 10.3 ± 0.1 and that CHO M1 membranes contained 580 ± 67 fmol/mg protein of receptor with affinity (−log Kd) of 9.7 ± 0.007 (n = 3; figs. 1A and 1B). Forty micrograms of membrane proteins and 0.18 nm [3H]QNB yielded specific binding rates of 92% for the M2 muscarinic receptor and 89% for the M3 muscarinic receptor.

In membranes prepared from cells expressing either the M2 or the M1 muscarinic receptor, carbachol displaced [3H]QNB with two affinity sites, which is expected for agonists that bind to G protein-coupled receptors (data not shown). These two affinity sites represent the receptor associated with the G protein (high affinity) and receptor dissociated from the G protein (low affinity). Chinese hamster ovary cells expressing either the M2 or the M1 muscarinic receptor showed a biphasic displacement curve for the competitive bind-
Table 1. Binding Affinity (IC₅₀) of Neuromuscular Relaxants for M₂ and M₃ Muscarinic Receptors

<table>
<thead>
<tr>
<th>Muscle Relaxants</th>
<th>M₂</th>
<th>M₃</th>
<th>[Peak Serum]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancuronium</td>
<td>0.28</td>
<td>1.2</td>
<td>4¹⁴</td>
</tr>
<tr>
<td>Succinylcholine</td>
<td>174</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Mivacurium</td>
<td>17</td>
<td>23</td>
<td>1.4¹⁵</td>
</tr>
<tr>
<td>Doxacurium</td>
<td>39</td>
<td>145</td>
<td>0.3¹⁶</td>
</tr>
<tr>
<td>Atracurium</td>
<td>5.0</td>
<td>1.4</td>
<td>1.3¹⁷</td>
</tr>
<tr>
<td>Rocuronium</td>
<td>3.0</td>
<td>19</td>
<td>4.7¹⁴</td>
</tr>
<tr>
<td>Gallamine</td>
<td>0.59</td>
<td>28</td>
<td>81¹⁸</td>
</tr>
<tr>
<td>Pipoxurinuim</td>
<td>5.8</td>
<td>13</td>
<td>3.5¹⁴</td>
</tr>
</tbody>
</table>

* Values are expressed as µM and are the mean values measured in at least three experiments for each neuromuscular relaxant.

The relative order of potency for the M₃ muscarinic receptor (highest to lowest) was pancuronium, atracurium, pipoxurinuim, rocuronium, mivacurium, gallamine, succinylcholine, and doxacurium.

Discussion

The affinities of neuromuscular blocking agents for the M₂ and M₃ muscarinic receptors were characterized in an effort to relate the affinities of the neuromuscular relaxants for muscarinic receptors with their known clinical side effects. This study performed in cells with pure populations of either the M₂ or M₃ muscarinic receptor allowed affinity measurements without the

![M₂ Muscarinic Receptor Diagram](image)

Fig. 2. Representative competitive displacement of H-QNB by neuromuscular relaxants in membranes prepared from Chinese hamster ovary cells expressing the M₂ muscarinic receptor. H-QNB (0.18 nM; 3.6 × Kᵢ of M₂ receptor) was incubated with increasing concentrations of neuromuscular relaxants. Pancuronium had the highest affinity for the M₂ muscarinic receptor compared with rocuronium, mivacurium, pipoxurinuim, and doxacurium (top), and succinylcholine, atracurium, and gallamine (bottom).

Anesthesiology, V 88, No 3, Mar 1998
M3 MUSCARINIC RECEPTOR

Fig. 3. Representative competitive displacement of $^3$H-QNB by neuromuscular relaxants in membranes prepared from Chinese hamster cells expressing the M3 muscarinic receptor. $^3$H-QNB (0.18 nM; 1 $\times$ Kd of M3 receptor) was incubated with increasing concentrations of neuromuscular relaxants. Pancuronium had the highest affinity for the M3 muscarinic receptor compared with rocuronium, mivacurium, pipercuronium, and doxacurium (top) and succinylcholine, atracurium, and gallamine (bottom).

competing effect of additional muscarinic receptor subtypes in the same cell. This eliminated a potential difficulty, seen in previous studies of native tissues in which multiple muscarinic receptor subtypes were present, confounding individual subtype affinity measurements.9

All neuromuscular blocking agents evaluated in the current study had measurable affinities for both the M2 and M3 muscarinic receptors, but there was a wide variation in their affinities for both receptors. In general, the affinity measurements determined in the CHO M1 cells were similar to those reported in cardiac membranes, which are thought to predominantly express M2 muscarinic receptors. Less similarity was found between the affinities of muscle relaxants measured in the CHO M3 cells and those reported in the literature for tissues such as ileum. Although ileum was originally thought to contain a relatively pure M3 muscarinic population, a mixture of M2 and M1 muscarinic receptors is now known to exist, with a predominance of the M2 subtype.10 In several studies, no differences were found in the affinities of muscle relaxants for the muscarinic receptor in heart or ileum, which is likely due to the predominance of the M2 subtype in both tissues.2,11

The clinical side effects induced by neuromuscular blocking agents are thought to be caused by their interaction with muscarinic receptors in the heart and airway. Muscle relaxants can interact with the muscarinic receptor in two ways. They can bind directly to the ligand binding site with agonist or antagonist properties, or, alternatively, muscle relaxants can interact with another region of the receptor, which causes a change in the characteristics of the binding site thus causing a decrease in ligand binding (a negative allosteric effect, e.g., gallamine)12 or an increase in ligand binding (a positive allosteric effect, e.g., alcuronium).13 In the current study, no attempt was made to distinguish between competitive antagonism versus negative allosteric effects of the muscle relaxants, as the clinical effect of either interaction is the same: the muscle relaxant would decrease the binding of acetylcholine to the muscarinic receptor.

In the current study, pancuronium and gallamine had the highest affinities for the M2 muscarinic receptor, whereas pancuronium and atracurium had the highest affinities for the M1 muscarinic receptor. The affinities of pancuronium and gallamine for the M2 receptor measured in the current study in a stably transfected cell line are in accordance with previous studies performed using rat heart membranes that predominantly express M2 muscarinic receptors.2,14 Rocuronium, atracurium, and pipercuronium had lower affinities for the M2 muscarinic receptor than did pancuronium and gallamine. Data from the current study are also consistent with a study in rat atria in which rocuronium had a higher affinity for muscarinic receptors than pipercuronium (Kd = 13.6 and 27 $\mu$M, respectively).14 Atracurium in this study had an affinity for the M1 receptor similar to the affinity reported in rat atria.11 One expected clinical consequence of blockade of the cardiac M2 muscarinic receptor is tachycardia, which occurs clinically with gallamine and pancuronium but is not reported with the newer-generation muscle relaxants. The results of

Anesthesiology, V 88, No 3, Mar 1998

Downloaded From: http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931273/ on 06/19/2017
our study are consistent with this mechanism of muscle relaxant–induced tachycardia in that gallamine and pancuronium were found to have the highest affinities for the M1 muscarinic receptor, with affinity values within the range of serum concentrations achieved with clinical use. Pancuronium and gallamine had IC50 values for the M1 muscarinic receptor of 0.28 and 0.59 μM, respectively, which are 14- and 137-fold lower, respectively, than the concentrations of these muscle relaxants achieved with routine clinical use, suggesting high M1 muscarinic receptor blockade with these agents. The muscle relaxant with the next highest affinity for M1 muscarinic receptors was rocuronium, which had an affinity IC50 value of 3.0 μM—a value only 1.6-fold lower than the concentration achieved with clinical use of rocuronium. The other muscle relaxants evaluated had affinities substantially lower than the serum concentrations achieved with normal clinical use.

Okanlami et al. from our laboratory previously evaluated the interaction of the nondepolarizing muscle relaxants pancuronium, mivacurium, pipercuronium, and doxacurium at M2 and M3 receptors in guinea pig heart and lung in vivo and found that the rank order of potency of the affinities (highest to lowest) for the M2 muscarinic receptor was similar to that seen in the current study—pancuronium, pipercuronium, mivacurium, and doxacurium—but they were unable to establish a rank order of potency for the M3 muscarinic receptor.

The affinities of pancuronium and gallamine for the M3 and M1 muscarinic receptors measured in the current study in cells that express only M3 muscarinic receptors were lower than that reported for the rat ileum. This discrepancy is likely explained by the measurement of affinity values in a tissue with a mixed population of muscarinic receptor subtypes (rat ileum), as opposed to the current study in which affinity was measured in cells expressing only the M3 muscarinic receptor subtype.

Muscarinic receptor control of airway tone is complicated. Airway smooth muscle expresses both M2 and M1 muscarinic receptors, whereas parasympathetic postganglionic nerves express M3 muscarinic receptors. The net effect of neuromuscular relaxant–induced effects on airways depends on the relative blockade of M2 and M3 muscarinic receptors. Because M1 muscarinic receptor activation is associated with initiation of airway smooth muscle contraction, agents that are potent antagonists at the M1 muscarinic receptor should inhibit bronchoconstriction despite the M2 muscarinic receptor blockade and the increased release of acetylcholine from parasympathetic postganglionic nerves. Therefore, pancuronium, which is more potent than gallamine as an M2 antagonist, should not and clinically does not appear to be associated with bronchoconstriction because pancuronium is a potent M1 muscarinic receptor antagonist at doses in the clinical range.

The current study of relative affinities of neuromuscular relaxants for muscarinic receptors is not a direct analysis of the clinical side effects of these drugs; however, the results suggest a possible role of muscarinic receptors in the mechanism of muscle relaxant–induced tachycardia by drugs such as gallamine and pancuronium due to M2 muscarinic receptor blockade. Moreover, the simultaneous blockade of M2 and M1 muscarinic receptors in the airway by relaxants would also explain a relative lack of clinical effect on airway tone, because blockade of the M1 receptor on the airway muscle prevents constriction by the increased release of acetylcholine by blockade of M2 muscarinic receptors on parasympathetic nerves.

References

3. Fryer AD, MacIagan J: Pancuronium and gallamine are antagonists for pre- and post-junctional muscarinic receptors in the guinea pig lung. Naunyn-Schmiedebergs Arch Pharmacol 1987; 335:367–71

Anesthesiology. V 88, No 3, Mar 1998


