Additive Inhibition of Nicotinic Acetylcholine Receptors by Corticosteroids and the Neuromuscular Blocking Drug Vecuronium

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Background: Neuromuscular disorders associated with muscular weakness and prolonged paralysis are common in critically ill patients. Acute myopathy has been described in patients receiving a combination therapy of corticosteroids and nondepolarizing neuromuscular blocking drugs for treatment of acute bronchospasm. The cause of this myopathy is not fully established and may involve drug interactions that perturb neuromuscular transmission. To investigate the interaction of corticosteroids with neuromuscular blocking drugs, the authors determined the effects of methylprednisolone and hydrocortisone alone and in combination with vecuronium on fetal (γ-subunit containing) and adult (ε-subunit containing) subtypes of the muscle-type nicotinic acetylcholine receptor.

Methods: Functional channels were expressed in Xenopus laevis oocytes and activated with 1 μM acetylcholine. The resulting currents were recorded using a whole cell two-electrode voltage clamp technique.

Results: Both forms of the muscle-type acetylcholine receptor were potently inhibited by methylprednisolone and hydrocortisone, with concentrations producing 50% inhibition in the range of 400–600 μM and 1–2 μM, respectively. The corticosteroids produced noncompetitive antagonism of the muscle-type nicotinic acetylcholine receptor at clinical concentrations. Both receptor forms were also inhibited, even more potently, by vecuronium, with a concentration producing 50% inhibition in the range of 1–2 μM. Combined application of vecuronium and methylprednisolone showed additive effects on both receptor forms, which were best described by a two-site model, with each site independent.

Conclusions: The enhanced neuromuscular blockade produced when corticosteroids are combined with vecuronium may augment pharmacologic denervation and contribute to the pathophysiology of prolonged weakness observed in some critically ill patients. (Key words: Antagonists; corticosteroids; drug interaction; intensive care; paralysis; vecuronium.)

NEUROMUSCULAR disorders associated with muscular weakness and prolonged paralysis have been described in many critically ill patients. The differential diagnosis includes neuropathic disorders, such as critical illness polyneuropathy,1,2 and myopathic disorders, such as acute quadriplegic myopathy,3 or “blocking agent–corticosteroid myopathy,” seen in critically ill asthmatic persons.4–8 To facilitate mechanical ventilation and minimize barotrauma, neuromuscular blocking drugs often are used in these patients. Most are also treated with large doses of corticosteroids. In patients undergoing this combined treatment regimen for as little as 96 h, severe muscle weakness has developed necessitating prolonged (weeks or months) recovery time.3

Although the mechanism of prolonged weakness in intensive care patients has not been conclusively established, pharmacologic denervation of the muscle-type nicotinic acetylcholine receptor (nAChR), as described by Dodson et al.,9 may play a role in the evolution of this pathologic state. Functional denervation could be produced by intense pharmacologic inhibition of the nAChR at the neuromuscular junction. This denervation may produce long-lasting functional changes in the postsynaptic muscle, leading to prolonged weakness. The absence of sensory deficits in most patients with this type of prolonged paralysis supports this hypothesis.9,10

The nAChR consists of four different subunits assembled in a pentameric structure, creating a central ion-conducting pore.11 Two subtypes of the muscle nAChR

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have been described: the adult or junctional form (ε-nAChR), with the subunit composition of α₂βδε, and the fetal or extrajunctional form (γ-nAChR), comprising α₂βγδ subunits. The muscle nAChR has two agonist binding sites with different affinities for acetylcholine, which originally were thought to reside entirely within each of the two α subunits. Newer affinity labeling and expression studies in Xenopus oocytes have shown contributions by the γ (ε) and δ subunits to the agonist binding sites. It is, therefore, assumed that these acetylcholine binding sites are located at the interfaces of the α–γ (or α–ε) and α–δ subunits.

Inhibitors of the nAChR can be grouped into two functional categories: (1) competitive antagonists, which include nondepolarizing neuromuscular blocking drugs that display a high affinity for the acetylcholine binding site, and (2) noncompetitive antagonists, which inhibit through either channel blockade or allosteric modulation of channel activity. Endogenous steroids, for example, act noncompetitively on neuronal, muscle-type, and ganglionic nAChRs. The effects of clinically used synthetic corticosteroids on the muscle-type nAChR have not been studied extensively. Bouzat and Barrantes observed, in patch clamp recordings from cells transfected with the subunits of the nAChR, a 50% reduction in the duration of the open state of the acetylcholine receptor by 400 µM hydrocortisone.

No data regarding a combined effect of corticosteroids and neuromuscular blocking drugs on the acetylcholine receptor are available. To evaluate the mechanism of inhibition of synthetic corticosteroids and nondepolarizing neuromuscular blocking drugs on the nAChRs, we heterologously expressed them in Xenopus oocytes and studied the discrete and combined effects of two corticosteroids (methylprednisolone and hydrocortisone) with vecuronium, a nondepolarizing muscle relaxant frequently used in the intensive care setting.

**Materials and Methods**

**Expression of Nicotinic Acetylcholine Receptor in Xenopus Oocytes**

Expression plasmids pSPα1, pGEMβ, pSPγ, and pSPδ encoding complementary DNA coding sequences for mouse muscle nAChR subunits α, β, γ, and δ, respectively, were provided by Drs. John Forsayeth and Zach Hall (Department of Physiology, University of California, San Francisco, CA). Expression plasmid pSPε was a gift from Dr. Paul Gardner (Department of Biochemistry, Dartmouth Medical School, New Hampshire, MA). These plasmids contain an SP6 promoter 5’ to the translation start codon that allows in vitro synthesis of RNA that directs the translation of each subunit. Complementary RNA (cRNA) for each cloned subunit was transcribed from the plasmids using the method of Melton. Aliquots of each cRNA synthesized from plasmids encoding the α, β, γ, and δ subunits were diluted 1:1.000 in ribonuclease-free water and mixed in the ratio of 2:1:1:1, respectively. When the ε subunit was substituted for the γ subunit, less dilution of the ε transcript (1:20) for combination with 1:1.000 diluted α, β and δ was necessary to produce similar levels of functional receptor expression.

The procedures for harvesting oocytes from the South African clawed frog (Xenopus laevis) were approved by the Committee on Animal Research of the University of California, San Francisco, and are similar to those previously described. Briefly, adult female frogs were anesthetized in 0.3% tricaine at 4°C, and unfertilized oocytes were removed through a small incision in the abdominal wall. After removal, oocytes were incubated with gentle agitation in high–magnesium-containing Ringer’s lactate (OR-Mg) solution (composition: 82 mM NaCl, 2 mM KCl, 5 mM HEPES, and 20 mM MgCl₂; pH 7.4) with 2 mg/ml collagenase A (Boehringer Mannheim, Indianapolis, IN) at room temperature. Oocytes were then washed twice with enzyme-free OR-Mg, twice with modified Barth’s solution with HEPES (MBSH; composition: 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 7 mM NaHCO₃, 1 mM CaCl₂, 1 mM Ca(NO₃)₂; pH 7.0), and mature oocytes (stages V and VI) were selected for injection. On the same day, oocytes were injected with mixtures (αβγδ or αβδε) of either diluted transcript or water as control. After injection, oocytes were maintained in modified Barth’s solution with HEPES (MBSH; composition: 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 7 mM NaHCO₃, 1 mM CaCl₂, 1 mM Ca(NO₃)₂; pH 7.0), and mature oocytes (stages V and VI) were selected for injection. On the same day, oocytes were injected with mixtures (αβγδ or αβδε) of either diluted transcript or water as control. After injection, oocytes were maintained in modified Barth’s solution with 50 mg/ml gentamycin, 2.5 mM sodium pyruvate, 5% heat-inactivated horse serum, and 5 mM theophylline at 18°C with gentle rotation.

**Two-electrode Voltage Clamp Recordings**

All electrophysiologic experiments were performed at room temperature (21–23°C) 1–3 days after oocyte injection. Acetylcholine receptor–mediated currents were measured by two-electrode voltage clamp (Axoclamp 2A; Axon Instruments, Foster City, CA). Microelectrodes were backfilled with 5 mM KCl and had resistances of 0.3–1.5 MΩ. All two-electrode voltage clamp experiments were performed using modified Barth’s solution as the perfusate. Recordings were obtained in a 25-µl recording chamber at flow rates of 3–5 ml/min. Water-injected oocytes were used as controls. All signals were
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filtered using an 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA) set at a 40-Hz cutoff before sampling at 100 Hz. Resulting signals were digitized and stored on a Power Macintosh 7100 (Apple Computer, Cupertino, CA) using data acquisition software (MacLab; ADInstruments, Milford, MA).

Methylprednisolone and hydrocortisone were obtained from the hospital pharmacy as sodium succinate salts (Pharmacia & Upjohn, Kalamazoo, MI) in preparations for clinical use. Both pharmaceutical preparations contain benzyl alcohol as preservative. Vecuronium also was obtained in preparations for clinical use (Marsam Pharmaceuticals Inc., Cherry Hill, NJ). Acetylcholine was purchased from Sigma (St. Louis, MO). Methylprednisolone, hydrocortisone, and vecuronium were prepared in modified Barth’s solution immediately before the experiments and diluted to the experimental concentrations. Oocytes with resting membrane potentials between −30 and −60 mV were studied. Channel opening was recorded as the negative steady state inward current necessary to maintain the voltage clamp during application of acetylcholine.

**Experimental Design**

Initial voltage-clamp experiments determined the concentration–response relation for acetylcholine for each subtype of the receptor. Further voltage-clamp experiments determined then the concentration–response relation for inhibition of 1 μM acetylcholine-induced currents by methylprednisolone and hydrocortisone. The relatively low agonist concentration of 1 μM acetylcholine was used to avoid desensitization of the fast or slow type of the receptors during repeated antagonist and agonist applications. A reduced number of openings per activation period and long closed times typically observed in the desensitization phenomenon have been shown in single channel recordings of the nAChR at higher acetylcholine concentrations in the presence of hydrocortisone.25 Atropine (0.5 μM) was present in all studies to prevent activation of an endogenous oocyte muscarinic receptor.26 Perfusion of the test drugs onto the oocytes was begun 30 s before a 10-s coapplication of acetylcholine and the test drug. The response was quantitated at the end of the acetylcholine application. A washout period of at least 90 s was ensured between each drug application to minimize the amount of desensitization during the course of an experiment. Each concentration was tested in more than three oocytes from different oocyte batches. The control response to 1 μM acetylcholine was repeated after washout of the inhibitory drug and averaged with the control response that preceded drug application to determine the “average control current.” Results were calculated as percent inhibition from the average control current as defined in equation 1.

\[
\% \text{ inhibition} = 100 \times \left(1 - \frac{\text{current in presence of agent}}{\text{average control current}}\right)
\]

After determining the concentration–response curves for methylprednisolone and hydrocortisone, the mechanism of inhibition by methylprednisolone was explored by applying increasing concentrations of the agonist acetylcholine in the presence of methylprednisolone.

Because benzyl alcohol is used as a preservative in the vials containing methylprednisolone and hydrocortisone and alcohols are known to affect the nAChR function,27 we performed control studies with benzyl alcohol, using the same protocol applied for the inhibitor studies.

A second series of experiments determined the concentration–response relation of vecuronium for both subtypes of the nAChR. As in the corticosteroid series, perfusion of vecuronium was begun 30 s before the application of acetylcholine, followed by a washout period of at least 90 s before reapplication of the agonist to determine the control response. The nature of the inhibition of nAChR currents by vecuronium was further evaluated by varying the agonist concentration in the presence and absence of the vecuronium concentration providing 50% inhibition (IC50).

A final set of experiments explored the effects of coapplication of different concentrations of methylprednisolone and vecuronium on the function of each subtype of the nAChR. A few experiments were performed with a higher agonist concentration (10 μM acetylcholine), in which the peak currents were measured as response.

**Statistical Analysis**

Data points were analyzed for acetylcholine and for each inhibiting drug, and the concentration–response relations were fitted to a logistic function by means of an iterative, nonlinear least-squares program, as described previously.28 To test for significant differences between inhibition of the two receptor subtypes or between the two steroids, analysis of covariance (ANCOVA) was used (JMP; SAS Institute Inc., Cary, NC). \(P < 0.05\) defined statistical significance. Results are mean ± SD. Different mathematical models for inhibition by competitive and noncompetitive inhibitors were fitted to the experimental data (see Appendix).
Results

Acetylcholine at different concentrations was applied for 10 s to voltage-clamped (−60 mV) oocytes that expressed either the fetal (α2βγδ) or the adult (α2βδε) subtype of the acetylcholine receptor. Acetylcholine elicited concentration-dependent inward currents. The data were fitted to the logistic equation. Maximal effect on both receptor forms was achieved with approximately 300 μM acetylcholine. The acetylcholine concentrations producing 50% of the maximal response (EC50) were similar for fetal (EC50: 12 μM; fig. 1) and for adult (EC50: 18 μM) muscle nAChRs.

Effects of Methylprednisolone and Hydrocortisone on the Nicotinic Acetylcholine Receptor

Methylprednisolone and hydrocortisone produced reversible, concentration-dependent inhibition of acetylcholine-induced currents in concentrations ranging in hundreds of μM (fig. 2). At high concentrations (≥5 mM), methylprednisolone and hydrocortisone completely inhibited agonist-induced currents. Control studies with benzyl alcohol concentrations up to 0.034%
showed no effect on nAChR function (data not shown).

The concentration-dependence of inhibition in each subtype of muscle nAChR is shown in figure 3. Fetal and adult subtypes of the nAChR both were equally sensitive to inhibition by methylprednisolone \([IC_{50}(MP) = 5.448 \text{ mM} \text{ for } \gamma\text{-nAChR}; IC_{50}(MP) = 5.570 \text{ mM} \text{ for } \epsilon\text{-nAChR})\] or hydrocortisone \([IC_{50}(HC) = 9.21 \text{ mM} \text{ for } \gamma\text{-nAChR}; IC_{50}(HC) = 1.290 \text{ mM} \text{ for } \epsilon\text{-nAChR})\]. Methylprednisolone was significantly more potent than hydrocortisone in producing inhibition of acetylcholine-induced currents (\(P < 0.05\)).

The inhibitory effect of methylprednisolone on \(\gamma\) and \(\epsilon\text{-nAChR} \) was studied in more detail. Inward currents evoked from nAChRs at various acetylcholine concentrations, in the absence and presence of 500 \(\mu\text{M}\) methylprednisolone (approximate IC\(_{50}\) with 1 \(\mu\text{M}\) acetylcholine), are shown in figure 4A. Peak inward currents during control conditions were dependent on acetylcholine concentration. Doses of 50 and 500 \(\mu\text{M}\) methylprednisolone both reduced nAChR-mediated inward currents at all acetylcholine concentrations studied. Increasing concentrations of acetylcholine were not able to overcome the inhibition produced by the steroid (fig. 4B).

**Effects of Vecuronium on the Nicotinic Acetylcholine Receptor**

Vecuronium, in concentrations in the nanomolar range, produced potent inhibition of both subtypes of the nAChR. Each receptor subtype was equally sensitive to inhibition by vecuronium \([IC_{50}(VEC) = 1.9 \text{ nM} \text{ for } \gamma\text{-nAChR}; IC_{50}(VEC) = 2.8 \text{ nM} \text{ for } \epsilon\text{-nAChR} \); fig. 5]. The Hill
slopes \( n \) of vecuronium-induced inhibition \( (n = 0.8 \text{ for } \gamma-nAChR \text{ and } 0.8 \text{ for } \varepsilon-nAChR) \) were smaller than the Hill slopes determined for either of the corticosteroids \( [n(MP) = 1.4 \text{ for } \gamma-nAChR \text{ and } = 1.8 \text{ for } \varepsilon-nAChR; \ n(HC) = 1.1 \text{ for } \gamma-nAChR \text{ and } = 1.2 \text{ for } \varepsilon-nAChR] \). Furthermore, at increasing acetylcholine concentrations, there was a significant decrease of the inhibition of nAChR currents produced by the IC\(_{50}\) of vecuronium. At an agonist concentration of 1 \( \mu M \) acetylcholine, 2 nm vecuronium produced an inhibition of 49 \( \pm \) 4\% \( (n = 4) \), whereas at 100 \( \mu M \) acetylcholine, the inhibition was only 10 \( \pm \) 4\% \( (n = 4; \text{ fig. 5B}) \), consistent with a competitive inhibition.

**Effects of Coapplication of Methylprednisolone and Vecuronium on the Nicotinic Acetylcholine Receptor**

To explore the combined effects of corticosteroids and the nondepolarizing neuromuscular blocking drug vecuronium on the nAChR function, we coapplied methylprednisolone and vecuronium in different concentrations to both receptor subtypes. Figure 6 shows representative tracings of one of these experiments: 10 \( nM \) vecuronium inhibited nAChR currents by 68\%, and 250 \( \mu M \) methylprednisolone inhibited the currents by 28\%; coadministration produced greater inhibition (83\%) than administration of either compound alone. The results of these coadministration experiments are summarized in table 1. The enhanced inhibition produced by a combination of these drugs also was observed when a higher agonist concentration was used. At 10 \( \mu M \) acetylcholine, 8 \( nM \) vecuronium inhibited nAChR currents by 68\%, and 250 \( \mu M \) methylprednisolone inhibited the currents by 28\%; coadministration produced greater inhibition (83\%) than administration of either compound alone. The results of these coadministration experiments are summarized in table 1. The enhanced inhibition produced by a combination of these drugs also was observed when a higher agonist concentration was used. At 10 \( \mu M \) acetylcholine, 8 \( nM \) vecuronium inhibited the nAChR peak currents by 37 \( \pm \) 2\% \( (n = 4) \) and 50 \( \mu M \) methylprednisolone inhibited the currents by 43 \( \pm \) 4\% \( (n = 4) \). Combined, 8 \( nM \) vecuronium and 50 \( \mu M \) methylprednisolone inhibited 10-\( \mu M \) acetylcholine-induced currents by 59 \( \pm \) 4\% \( (n = 4) \).

To characterize the nature of the interaction between methylprednisolone and vecuronium at 1 \( \mu M \) acetylcholine as an agonist concentration, we estimated, in addition to IC\(_{50}\) values and Hill coefficients, possible interaction terms. We fitted different models of drug interaction (equations 4-7 in the Appendix) to the observed data points for the \( \gamma-nAChR \) \( (n = 115) \) and \( \varepsilon-nAChR \) \( (n = 58) \). Each treatment combination represents inhibition values obtained in the same oocyte. Tables 2 and 3 summarize the results of the mathematical modeling. For the \( \gamma-nAChR \) (table 2), the model with the lowest Akaike information criterion (AIC; see equation 8 in the Appendix),\(^{29}\) indicating the best model fit, showed no interac-

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**Fig. 4. Effect of methylprednisolone on the concentration-response of acetylcholine on the \( \gamma \) subtype of the nicotinic acetylcholine receptor (nAChR).** (A) Inward currents evoked by superfusion of 1, 10, and 100 \( \mu M \) acetylcholine (ACh) alone (as indicated by horizontal bars) during control condition and during coapplication of 500 \( \mu M \) methylprednisolone. Methylprednisolone was preapplied for 30 s before coapplication with 1, 10, and 100 \( \mu M \) acetylcholine. Complete reversal of the effect was observed after 2 or 3 min of washing (data not shown). (B) The effect on inward currents mediated by the \( \gamma \) subtype of the nAChR over a range of acetylcholine concentrations in the presence of 0, 50, and 500 \( \mu M \) methylprednisolone (MP). Data points represent mean \( \pm \) SD (error bars) of three to six oocytes.
tion between the effects of the two drugs (equation 5 Appendix; AIC = 76). For the e-nAChR (table 3), the model fit was only slightly improved by the introduction of an interaction term σ (equation 6; Appendix; AIC = −26). For both the γ-nAChR and the e-nAChR, a model involving cooperative binding of both agents (equation 7; Appendix) failed to improve the fit.

Discussion

The potency of the neuromuscular blocking drug vecuronium and the synthetic corticosteroids hydrocortisone and methylprednisolone at the nAChR is clinically important because patients presenting with prolonged-muscular weakness often receive both agents as part of

Fig. 5. (A) Concentration–response effect for vecuronium for inhibition of acetylcholine-induced (1 μM) inward currents mediated by the γ subtype and e subtype of the nicotinic acetylcholine receptor (nAChR). Smooth curves represent least-squares fit of logistic equation as described in Methods. Data points show mean ± SD (error bars) of three to eight oocytes. (B) Inhibition of the currents by 2 nM vecuronium can be overcome with increasing acetylcholine concentrations. Data points represent mean ± SD (error bars) of four oocytes.

Fig. 6. Effect of 10 nM vecuronium and 250 μM methylprednisolone alone and in combination on acetylcholine-induced (1 μM) inward currents mediated by the γ subtype of the nicotinic acetylcholine receptor (nAChR). Horizontal bars indicate drug applications. Methylprednisolone, vecuronium, and the combination of both drugs were preapplied for 30 s before coapplication with 1 μM acetylcholine. After each drug application, a 1-μM acetylcholine-induced inward current was obtained as a control (last control not shown).

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formed by Bouzat and Barrantes evaluated the effects of hydrocortisone on the muscle-type acetylcholine receptor at a single channel level. They found a 50% inhibition by 400 μM hydrocortisone and concluded that an inhibitory effect on the acetylcholine receptor occurs during treatment with doses of hydrocortisone as low as 100 mg. Cortisol levels as high as 500 μM also have been measured in septic patients, who often present with neuromuscular abnormalities. These clinical studies report steroid concentrations in human plasma, in which part of the steroid is protein-bound, whereas the steroid concentrations reported in the current study are in serum-free buffer.

In our study, methylprednisolone inhibited both subtypes of the nAChR approximately 2 or 3 times more potently than did hydrocortisone. Our results also show that block of nAChR function by methylprednisolone was insurmountable by increasing concentrations of acetylcholine, implying a noncompetitive type of inhibition. It has been shown that noncompetitive inhibitors can increase the allosteric constant, thereby increasing the rate of onset of desensitization and increasing the affinity for the agonist. We believe that the increased inhibition (90%) produced by 500 μM methylprednisolone at a higher agonist concentration is also caused by greater desensitization of the receptor. Therefore, the IC50s for steroids administered for this study are dependent on the agonist concentration (1 μM). Radioligand-binding studies showed that steroids act noncompetitively at extracellular sites to inhibit ganglionic nAChR function. Combined with the finding of previous studies, our findings suggest that methylprednisolone and hydrocortisone also act as noncompetitive inhibitors of both subtypes of the muscle nAChR.

In contrast, previous work in the 1970s, performed to clarify the therapeutic effects of corticosteroids in myasthenia gravis patients, found that corticosteroids may improve muscle performance. However, a recent study by Bouzat and Barrantes showed that fetal and adult nAChRs, and the mutant nAChR associated with a myasthenic syndrome, are affected in a similar manner by the corticosteroid hydrocortisone, which acts as a noncompetitive inhibitor of all receptor subtypes.

**Vecuronium**

The inhibition of both muscle nAChR subtypes by vecuronium was very potent, with IC50 values in the nanomolar range. These values are consistent with previously reported potency values for pancuronium. Interestingly, in this previous study, pancuronium caused significantly more potent inhibition of γnAChR (IC50 = 300 μM acetylcholine) than the EC50 of acetylcholine.

### Table 1. Percent Inhibition of Acetylcholine-induced (1 μM) Currents Mediated by the γ Subtype of the Nicotinic Acetylcholine Receptor by Vecuronium, Methylprednisolone, and Combinations of Both Drugs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vecuronium 300 pm</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Methylprednisolone 500 μM</td>
<td>42 ± 11</td>
</tr>
<tr>
<td>Combined experiment</td>
<td>55 ± 12</td>
</tr>
<tr>
<td>Vecuronium 1 nM</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>Methylprednisolone 500 μM</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Combined experiment</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>Vecuronium 1 nM</td>
<td>25 ± 10</td>
</tr>
<tr>
<td>Methylprednisolone 250 μM</td>
<td>32 ± 10</td>
</tr>
<tr>
<td>Combined experiment</td>
<td>47 ± 16</td>
</tr>
<tr>
<td>Vecuronium 10 nM</td>
<td>67 ± 6</td>
</tr>
<tr>
<td>Methylprednisolone 250 μM</td>
<td>41 ± 16</td>
</tr>
<tr>
<td>Combined experiment</td>
<td>73 ± 6</td>
</tr>
<tr>
<td>Vecuronium 10 nM</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>Methylprednisolone 750 μM</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>Combined experiment</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>Vecuronium 1 nM</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Methylprednisolone 750 μM</td>
<td>57 ± 7</td>
</tr>
<tr>
<td>Combined experiment</td>
<td>60 ± 4</td>
</tr>
</tbody>
</table>

Values are mean ± SD. For each experiment, n = 3.

their treatment regimen. First, we established the action of acetylcholine on the subtypes of the nicotinic receptor. The maximal effect and EC50 values of acetylcholine were in close agreement with previously published data and did not differ significantly between the two subtypes. The expected agonist concentration at the neuromuscular endplate is thought to be much higher (300 μM acetylcholine) than the EC50 of acetylcholine observed in oocytes (20 μM). Because desensitization of the nAChR has been described with acetylcholine concentrations as low as 10–50 μM, we used 1 μM acetylcholine as the agonist for most of our study to ensure reproducible, nondesensitized responses.

**Corticosteroids**

In the oocyte expression system, methylprednisolone and hydrocortisone produced concentration-dependent inhibition of nAChR-mediated inward currents. The concentrations of the steroids that produced inhibition were in the range of hundreds of micromolar, overlapping the concentrations measured in patients. For example, Defer et al. reported concentrations greater than 100 μM in patients who received methylprednisolone for acute exacerbation of multiple sclerosis. Previous work performed by Bouzat and Barrantes evaluated the effects of methylprednisolone and hydrocortisone, which acts as a noncompetitive inhibitor of all receptor subtypes.
Table 2. Summary of Mathematical Models for Antagonist Inhibition of the γ-type Nicotinic Acetylcholine Receptor

<table>
<thead>
<tr>
<th>Models for Antagonist Inhibition</th>
<th>P</th>
<th>IC50(VEC) (nM)</th>
<th>IC50(MP) (μM)</th>
<th>n(VEC)</th>
<th>n(MP)</th>
<th>Interaction Term</th>
<th>SS R</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>R = \frac{IC_{50(VEC)}}{VEC} + \frac{IC_{50(MP)}}{MP}</td>
<td>2</td>
<td>2.5</td>
<td>461</td>
<td>1*</td>
<td>1*</td>
<td>—</td>
<td>1.94</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.4</td>
<td>482</td>
<td>1*</td>
<td>1.4</td>
<td>—</td>
<td>1.87</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.5</td>
<td>489</td>
<td>0.8</td>
<td>1.4</td>
<td>—</td>
<td>1.81</td>
<td>76†</td>
</tr>
<tr>
<td>R = \frac{IC_{50(VEC)}}{VEC} + \frac{IC_{50(MP)}}{MP} + (\sigma \times VEC \times MP)</td>
<td>3</td>
<td>2.2</td>
<td>440</td>
<td>1*</td>
<td>1*</td>
<td>NS</td>
<td>1.96</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.9</td>
<td>423</td>
<td>1*</td>
<td>1.5</td>
<td>NS</td>
<td>2.01</td>
<td>88</td>
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<tr>
<td></td>
<td>5</td>
<td>2.2</td>
<td>474</td>
<td>0.9</td>
<td>1.4</td>
<td>NS</td>
<td>1.83</td>
<td>79</td>
</tr>
<tr>
<td>R = \frac{IC_{50(VEC)}}{VEC} + \frac{IC_{50(MP)}}{MP} + (\frac{I_{50}}{I_{50} + (VEC \times MP)})</td>
<td>3</td>
<td>2.5</td>
<td>461</td>
<td>1*</td>
<td>1*</td>
<td>∞</td>
<td>1.94</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.4</td>
<td>482</td>
<td>1*</td>
<td>1.4</td>
<td>∞</td>
<td>1.87</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.5</td>
<td>489</td>
<td>0.8</td>
<td>1.4</td>
<td>∞</td>
<td>1.81</td>
<td>78</td>
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</tbody>
</table>

IC\(_{50}\), Hill coefficients, and interaction terms were obtained from fitting the different equations in the Appendix to the experimental data (n = 115).

IC\(_{50}\) = concentration producing 50% inhibition; VEC = vecuronium; MP = methylprednisolone; n = Hill coefficient; \(\sigma\) and \(I_{50}\) = estimated interaction terms; P = number of estimated parameters; SS\(_{R}\) = residual sum of squares of the corresponding model fit; AIC = Akaike information criterion, calculated according to equation (8) in the Appendix; NS = nonsignificant.

* Fixed values in model fit.
† Model with best fit according to AIC.

1.7 nM) than of e-nAChR (IC\(_{50}\) = 46 nM), whereas potency of inhibition with atracurium, a curariform muscle relaxant, was the same for both subtypes. The Hill coefficient describing the concentration–response relation of vecuronium was close to unity for both subtypes, suggesting that vecuronium inhibits γAChR and e-nAChR at a single site. It is widely accepted that the major mechanism of action of neuromuscular blocking drugs is competitive inhibition of activation. The finding of a Hill coefficient close to unity also is in agreement with previous work that shows two acetylcholine binding sites with different affinities for d-tubocurarine. Similar Hill coefficients (range, 0.7–0.9) have been reported for other neuromuscular blocking drugs. The experiments

Table 3. Summary of Mathematical Models for Antagonist Inhibition of the ε-type Nicotinic Acetylcholine Receptor

<table>
<thead>
<tr>
<th>Models for Antagonist Inhibition</th>
<th>P</th>
<th>IC50(VEC) (nM)</th>
<th>IC50(MP) (μM)</th>
<th>n(VEC)</th>
<th>n(MP)</th>
<th>Interaction Term</th>
<th>SS R</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R = \frac{IC_{50(VEC)}}{VEC} + \frac{IC_{50(MP)}}{MP}</td>
<td>2</td>
<td>3.1</td>
<td>615</td>
<td>1*</td>
<td>1*</td>
<td>—</td>
<td>0.83</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.9</td>
<td>591</td>
<td>1*</td>
<td>1.8</td>
<td>—</td>
<td>0.64</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.1</td>
<td>596</td>
<td>0.8</td>
<td>1.8</td>
<td>—</td>
<td>0.59</td>
<td>23</td>
</tr>
<tr>
<td>R = \frac{IC_{50(VEC)}}{VEC} + \frac{IC_{50(MP)}}{MP} + (\sigma \times VEC \times MP)</td>
<td>3</td>
<td>2.7</td>
<td>576</td>
<td>1*</td>
<td>1*</td>
<td>NS</td>
<td>0.78</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.6</td>
<td>569</td>
<td>1*</td>
<td>1.7</td>
<td>NS</td>
<td>0.61</td>
<td>21</td>
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<tr>
<td></td>
<td>5</td>
<td>2.7</td>
<td>569</td>
<td>0.7</td>
<td>1.7</td>
<td>−0.0003</td>
<td>0.54</td>
<td>26†</td>
</tr>
<tr>
<td>R = \frac{IC_{50(VEC)}}{VEC} + \frac{IC_{50(MP)}}{MP} + (\frac{I_{50}}{I_{50} + (VEC \times MP)})</td>
<td>3</td>
<td>3.1</td>
<td>615</td>
<td>1*</td>
<td>1*</td>
<td>∞</td>
<td>0.83</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.9</td>
<td>591</td>
<td>1*</td>
<td>1.8</td>
<td>∞</td>
<td>0.64</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.1</td>
<td>596</td>
<td>0.8</td>
<td>1.8</td>
<td>∞</td>
<td>0.59</td>
<td>21</td>
</tr>
</tbody>
</table>

IC\(_{50}\), Hill coefficients, and interaction terms were obtained from fitting the different equations in the Appendix to the experimental data (n = 58).

IC\(_{50}\) = concentration producing 50% inhibition; VEC = vecuronium; MP = methylprednisolone; n = Hill coefficient; \(\sigma\) and \(I_{50}\) = estimated interaction terms; P = number of estimated parameters; SS\(_{R}\) = residual sum of squares of the corresponding model fit; AIC = Akaike information criterion, calculated according to equation (8) in the Appendix; NS = nonsignificant.

* Fixed values in model fit.
† Model with best fit according to AIC.

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using the IC$_{50}$ of vecuronium (2 nM) and increasing acetylcholine concentrations up to 500 μM showed that the inhibition of vecuronium could be overcome by increasing agonist concentration, confirming that vecuronium acts in a competitive manner (fig. 5B).

Combined Effects of Methylprednisolone and Vecuronium on the Nicotinic Acetylcholine Receptor

To determine whether corticosteroids and nondepolarizing neuromuscular blocking drugs have an interactive effect, we analyzed different models of inhibition produced by combined exposure, considering dependent and independent interactions between the binding sites for the two classes of inhibitors. For the γ-nAChR, the model with the lowest AIC$_{29}$ was described by equation 6 (Appendix, table 3); that is, this interaction term was small and differed significantly from zero only when the Hill coefficients of both inhibitors also were estimated. In addition, using this interaction term σ changed the predicted response of a combined experiment only by approximately 3%. For both the γ-nAChR and the e-nAChR, the model involving cooperative binding of both inhibitors described by equation 7 failed to improve the fit because it estimated a very large IC$_{50}$ (essentially ∞; see Appendix, equation 7), resulting in unity for the interaction term

\[
\frac{IC_{50}}{IC_{50} + (VEC \times MP)}
\]

Therefore, we conclude that the combined effects of vecuronium and methylprednisolone are independent and additive for either subtype of the nAChR as described by equation 5 in the Appendix.

The motivation for initiating these studies was to identify actions on the nAChR that may contribute to the pathologic state of prolonged weakness in intensive care patients. Initial case reports reported rapidly developing muscle weakness in patients with severe bronchospasm when nondepolarizing neuromuscular blocking drugs were administered concurrently with high-dose corticosteroids. The mechanism of this rapidly developing weakness remains unclear. Electromyographic studies showed normal motor and sensory nerve conduction, but motor-evoked potentials and compound action potentials showed reduced amplitudes. These findings suggest an abnormality in the neuromuscular junction. In rats exposed solely to high doses of steroids, histologic examinations revealed atrophy with a normal muscular pattern. When Rouleau et al. denervated rat hind limbs they found that the denervated muscle was markedly more atrophied after 7 days if corticosteroids were administered. These findings suggest that denervation makes the neuromuscular junction and skeletal muscle hypersensitive to steroid-induced myopathy. These authors concluded that corticosteroids and denervation lead to a reduced synthesis of myosin, primarily by reducing the numbers of ribosomes. In addition, Wokke et al. found in their histologic investigation in critically ill patients with generalized weakness only minor axonal neuropathy and concluded that pharmacologic denervation may explain, at least partially, the histologic abnormalities. Denervation, whether mechanistic or pharmacologic, appears to increase sensitivity to corticosteroid-induced myocyte catabolism.

The duration of the clinically observed weakness in these critically ill patients may last weeks or months, long after neuromuscular blocking drugs are undetectable in the serum. This prolonged weakness occurs almost exclusively in patients who were treated with a combination of neuromuscular blocking drugs and corticosteroids. Initial studies of patients with prolonged paralysis who were treated with vecuronium during intensive care suggested that accumulation of metabolites such as 3-desacetylvecuronium may cause prolonged neuromuscular blockade. However, the persistence of symptoms long after metabolites were detectable in serum discounts the likelihood of metabolite accumulation because of abnormal elimination. Interestingly, in the aforementioned study, five of seven patients with prolonged paralysis received steroids during or shortly before administration of vecuronium as part of their treatment; however, in patients who showed no prolonged paralysis, only one of nine patients received steroids in addition to the neuromuscular blocking drug (V. Segredo, M.D., personal communication, unpublished data, January 1999).

Our study indicates no positive cooperation between the two classes of inhibitors—neuromuscular blocking drugs and corticosteroids—with respect to their effect on the function of the muscle-type nAChR. However, the findings that inhibition of both subtypes of the receptor by vecuronium and methylprednisolone appear to occur through actions at separate and independent sites and that this inhibition is pharmacologically additive may explain the enhanced neuromuscular blockade observed.
in some patients treated with a combination of these drugs. This combination may cause enhanced pharmacologic denervation, which then may lead to myopathy. That treatment—combining corticosteroids with neuromuscular blocking drugs—can result in prolonged weakness suggests the need for more research to improve our understanding of the cellular and molecular basis of this neuromuscular disorder.

The authors thank Dr. James E. Caldwell (Associate Professor, University of California, San Francisco, CA) for valuable discussion and Winifred von Ehrenburg for her expert editorial assistance.

Appendix: Mathematical Modeling of Antagonist Interaction

We began the process of mathematical modeling using a standard model for competitive and noncompetitive inhibition

\[ E = \frac{E_{\text{max}(A)} \times A}{A + EC_{50}(A) \times \left(1 + \frac{\text{VEC}}{IC_{50\text{VEC}}}ight) \times \left(1 + \frac{\text{MP}}{IC_{50\text{MP}}}ight)} \]  

(2)

where the response \( E \) is determined by the interaction of the agonist \( A \) (acetylcholine), the competitive antagonist vecuronium \( \text{VEC} \), and the noncompetitive antagonist methylprednisolone \( \text{MP} \). \( E_{\text{max}} \) is the maximal achievable effect by acetylcholine in the absence of vecuronium and methylprednisolone, and \( EC_{50} \) is the concentration of agonist obtaining half-maximal effect. This model formed the base from which we derived alternative models that were fitted to the experimental data.

Because the agonist concentration \( A \) was fixed to a concentration much lower than \( EC_{50} \), equation 2 reduces to

\[ E = \frac{E_{\text{max}} \times A}{EC_{50}(A) \times \left(1 + \frac{\text{VEC}}{IC_{50\text{VEC}}}ight) \times \left(1 + \frac{\text{MP}}{IC_{50\text{MP}}}ight)} \]  

(3)

Although vecuronium is a competitive antagonist and methylprednisolone is a noncompetitive antagonist, when the agonist concentration acetylcholine is much lower than \( EC_{50} \), the type of inhibition produced by vecuronium or methylprednisolone alone (competitive or noncompetitive) is not distinguishable. Using equation 3, the percent inhibition \( R \) (defined as the ratio of observed response at a given acetylcholine concentration with vecuronium or methylprednisolone concentrations or both greater than zero, divided by the observed response with zero concentrations of vecuronium and methylprednisolone) can be written as

\[ R = \frac{IC_{50\text{VEC}}}{\text{VEC} + IC_{50\text{VEC}}} \times \frac{IC_{50\text{MP}}}{\text{MP} + IC_{50\text{MP}}} \]  

(4)

Equation 4, where \( IC_{50\text{VEC}} \) and \( IC_{50\text{MP}} \) are the only parameters to be estimated, is the simplest model we considered to fit to the experimental data. Inclusion of Hill coefficients \( n \) for both the competitive and the noncompetitive antagonists produced a slightly more complex model:

\[ R = \frac{IC_{50\text{VEC}}}{\text{VEC} + IC_{50\text{VEC}}} \times \frac{IC_{50\text{MP}}}{\text{MP} + IC_{50\text{MP}}} \]  

(5)

To test for the presence of significant interaction between vecuronium and methylprednisolone, we considered two additional models. The first is an empirical model that extends equation 5 by the addition of \( \sigma \), an interaction term for vecuronium and methylprednisolone:

\[ R = \frac{IC_{50\text{VEC}}}{\text{VEC} + IC_{50\text{VEC}}} \times \frac{IC_{50\text{MP}}}{\text{MP} + IC_{50\text{MP}}} \times (\sigma \times \text{VEC} \times \text{MP}) \]  

(6)

The second was obtained by assuming a hypothetical site of action necessitating binding of vecuronium and methylprednisolone:

\[ R = \frac{IC_{50\text{VEC}}}{\text{VEC} + IC_{50\text{VEC}}} \times \frac{IC_{50\text{MP}}}{\text{MP} + IC_{50\text{MP}}} \times \text{I}_{50} \]  

(7)

where \( I_{50} \) expresses the concentration of vecuronium times methylprednisolone, at which half the inhibitory effect is achieved.

Equations 4–7 were fitted to the data of all experiments, with Hill coefficients for vecuronium and methylprednisolone fixed to 1, only the Hill coefficient of vecuronium fixed to 1, or estimates of both Hill coefficients. We used the nonlinear minimization program contained in the statistical software package JMP (SAS Institute Inc., Cary NC). The best model of \( \alpha \)ChR inhibition was selected using the Akaike information criterion (AIC):29

\[ \text{AIC} = N \ln SS_{n} + 2P \]  

(8)

where \( N \) is the number of data points, \( P \) is the number of estimated parameters, and \( SS_{n} \) is the residual sum of squares. This criterion for best model balances the model fit, as quantified by the residual sum of squares, against the number of estimated parameters. The models were further evaluated by plotting the residual errors to detect systematic deviation between data and model prediction, and by evaluating the plausibility of the estimated parameters.

References


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