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Carboetomidate Inhibits Alpha4/Beta2 Neuronal Nicotinic Acetylcholine Receptors at Concentrations Affecting Animals

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Abstract

Background—Carboetomidate is an etomidate derivative that produces hypnosis without inhibiting adrenal corticosteroid synthesis. Like etomidate, carboetomidate modulates γ -

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Conflicts of Interest: Dr. Raines is a co-inventor on patented drugs, including carboetomidate, and has an equity interest in Annovation Biopharma, which licenses these patents. MGH owns these patents. Dr. Raines, his lab, and MGH could realize income if the patent licenses or Annovation Biopharma are sold or generate revenue.

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Conflicts of Interest: Stuart A. Forman is a co-inventor of patented drugs, including carboetomidate. MGH owns these patents. Dr. Forman, his lab, and MGH could realize royalty income if the patents are licensed and generate revenue.

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aminobutyric acid type A receptors, but its effects on other ion channel targets of general anesthetics are unknown.

Methods—We compared etomidate and carboetomidate effects on human N-methyl-D-aspartate receptors or neuronal nicotinic receptors (nnAChRs) expressed in *Xenopus* oocytes, using two micro-electrode voltage clamp electrophysiology.

Results—Etomidate did not affect either type of receptor at clinically relevant concentrations, whereas carboetomidate concentrations near EC50 for anesthesia significantly inhibited nnAChRs.

Conclusions—Compared to etomidate, carboetomidate's higher hydrophobicity is associated with greater inhibition of nnAChRs.

Introduction

Carboetomidate is a new sedative hypnotic pyrrole with a molecular structure similar to etomidate.¹ The replacement of an imidazole nitrogen on etomidate with carbon increases hydrophobicity,² while modestly reducing hypnotic potency and eliminating inhibition of adrenocortical steroid synthesis. Etomidate is remarkably selective for ion channel targets, acting almost exclusively at γ -aminobutyric acid type A (GABA_A) receptors containing β 2 or β 3 subunits.^{3,4} Carboetomidate also modulates β 2/3 GABA receptors,¹ but its effects on other ion channel targets of general anesthetics remain unexplored.

Methods

Animals

Female *Xenopus laevis* frogs were housed in a veterinarian-supervised facility and used with approval from the Massachusetts General Hospital Subcommittee on Research Animal Care. Methods for *Xenopus* oocyte harvest and culture have been described previously.⁵

Ion channel expression

DNA plasmids encoding human N-methyl-d-aspartate (NMDA) receptor (NMDAR) subunits NR1B and NR2A were obtained from Prof. Steven Treistman (University of Massachusetts Medical School, Worcester, MA). Plasmids encoding the human neuronal nicotinic acetylcholine receptor (nnAChR) subunits α 4 and β 2 were obtained from Prof. James Patrick (Salk Institute, La Jolla, CA). Capped messenger RNAs (mRNAs) were transcribed in vitro using commercial kits (Ambion, Austin TX). Oocytes were injected with 1:1 mRNA mixtures of NR1B + NR2A or α 4 + β 2, and incubated in ND96 solution (in mM: 96 NaCl, 4 KCl, CaCl₂ 1.8, MgCl₂ 1.0, Hepes 5, and EGTA 1.0, pH 7.5) supplemented with 100 μ g/ml each of amikacin and ciprofloxacin⁶ for 48-96 hours before electrophysiology.

Drugs and chemicals

Etomidate (Bedford Laboratories, Bedford, OH) was formulated in 35% propylene glycol:water (v/v). Carboetomidate (Aberjona Laboratories, Beverly, MA) was prepared as a 10mM stock in dimethylsulfoxide (DMSO). Anesthetic stock solutions were diluted in appropriate electrophysiology buffer for each type of channel. Propylene glycol or DMSO, at the highest concentrations present in experiments, produced no effects on ion channels. Extra virgin olive oil (lot L-134-AB) was purchased from World Finer Foods, Inc. (Bloomfield, NJ). Acetylcholine chloride, NMDA, glycine, antibiotics, and other buffers and salts were purchased from Sigma-Aldrich (St. Louis, MO).

Partition coefficients

Partition coefficients between olive oil and aqueous buffer (Tris 10 mM, pH 7.4) were assessed at 20 to 22 °C using published methods based on quantitative high performance liquid chromatography.²

Electrophysiology

Two micro-electrode electrophysiology techniques for NMDARs and nnAChRs have been described previously.^{5,7} Voltage-clamped currents (20 to 22 °C) were digitized (100 Hz), recorded, and analyzed offline to evaluate baseline-corrected peaks. In nnAChR experiments, oocytes were pre-exposed to anesthetic for 30-90s before activation with 10 μM acetylcholine in the presence of anesthetic. Peak currents were normalized to responses measured in the same cell without anesthetic. For NMDAR experiments, receptors were first activated by agonists (100 μM NMDA plus 10 μM glycine) and then exposed to agonists plus anesthetic for a period of time before returning to agonists alone. Anesthetic effects were assessed by comparing currents averaged over a 10s epoch immediately before the addition of drug to a 10s epoch after full effect was evident.

Data analysis

Normalized responses at each anesthetic concentration were averaged for $n > 5$ oocytes, from at least two frogs, and reported as mean $\pm$ sd. Concentration-dependent data were fitted with logistic (Hill) equations of the form:

$$\frac{I^{\text{Drug}}}{I_{\text{max}}} = \frac{1}{1 + 10^{(\log[\text{Drug}] - \log[\text{IC}_{50}]) \cdot nH}} \quad \text{Equation 1}$$

using Graphpad Prism 5.1 (Graphpad.com; La Jolla, CA). IC_{50} is the drug concentration producing 50% inhibition and nH is the Hill slope. IC_{50} confidence intervals were calculated from fitted $\log[\text{IC}_{50}]$ and standard error values.

Results

Olive oil/buffer partition coefficients for etomidate and carboetomidate were measured in triplicate. Results for etomidate were 2472, 1783, and 1932 (2100 ± 360 ; mean $\pm$ sd) and for carboetomidate were 8202, 7926, and 6994 (7700 ± 630).

NMDA receptors are thought to contribute to immobility for various anesthetic drugs.⁸ Our initial electrophysiological studies examined the effect of high concentrations of anesthetics on NR1B/NR2A NMDA receptors (Fig. 1). Etomidate at 100 μM produced $15 \pm 8.9\%$ (mean $\pm$ sd; $n = 5$) inhibition of NMDARs, consistent with previous reports.^{9,10} Near-saturated solutions of carboetomidate in aqueous buffer (40 μM) inhibited NMDA receptors by $2.1 \pm 0.60\%$ ($n = 5$). Given that the concentrations tested are approximately eight times the carboetomidate EC_{50} (5.4 μM) and 43 times the etomidate EC_{50} (2.3 μM) for loss of righting reflexes (LORR) in tadpoles, we did not further examine the concentration-dependent effects of either drug in NMDARs.

Neuronal nAChRs apparently contribute little to immobility,^{11,12} but may partially mediate amnesia and other anesthetic actions. In oocytes expressing $\alpha 4\beta 2$ nnAChRs, we observed significant concentration-dependent inhibition of ACh-activated currents by carboetomidate (Fig 2). The carboetomidate IC_{50} for this effect was 13 μM (95% CI: 9.9 to 18 μM; $n = 5$). Etomidate inhibited nnAChRs with an IC_{50} of 160 μM (95% CI: 113 to 229 μM; $n = 6$). At their respective EC_{50} s for LORR in tadpoles,¹ our results predict more than 25% inhibition of $\alpha 4\beta 2$ nnAChRs by carboetomidate and less than 5% inhibition by etomidate.

Conclusions

Our major new finding is that carboetomidate, at concentrations near its EC₅₀ for LORR in animals, inhibits $\alpha 4\beta 2$ nnAChR channels, but not NR1B/NR2A NMDARs. In agreement with prior studies,^{9,10} we also observed that both human NMDARs and nnAChRs are insensitive to etomidate at clinically relevant concentrations. We measured oil:water partition coefficients for carboetomidate and etomidate, and found changes in comparison to n-octanol:water partition coefficients.² Interestingly, etomidate partitions from water more into olive oil than into n-octanol, while carboetomidate partitions more into n-octanol than into oil. As a result, the carboetomidate:etomidate ratio of partition coefficients in olive oil is 3.7, while in n-octanol this ratio is 18.8. The inhibitory potency of halogenated ether anesthetics and nonimmobilizers at $\alpha 4\beta 2$ nnAChRs is strongly dependent on the olive oil/water partition coefficient.⁵ Both etomidate and carboetomidate appear to follow this correlation, because inhibition of nnAChRs by carboetomidate is 12-fold more potent than etomidate. Thus, perhaps because carboetomidate has greater hydrophobicity but lower anesthetic potency than etomidate, equi-effective anesthetic concentrations of carboetomidate inhibit nnAChRs far more than etomidate.

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Figure 1. Inhibition of NR1B/NR2A n-methyl-D-aspartate receptors by etomidate and carboetomidate. Panel A: The molecular structure of etomidate. Panel B: The molecular structure of carboetomidate. The five-membered imidazole ring of etomidate is replaced by a pyrrole ring in carboetomidate. Panel C: A current trace recorded from an oocyte expressing n-methyl-D-aspartate (NMDA) receptors is shown. Agonist (NMDA plus glycine) application is depicted by the white bar above the current trace, and etomidate plus agonist application is depicted by the black bar. The agonist-activated current is reversibly inhibited by approximately 23% during 100 μ M etomidate application. Panel D: A current trace from another oocyte is shown, illustrating the effect of 40 μ M carboetomidate. The agonist activated current shown is inhibited by 3% during carboetomidate application.

Figure 2.

Inhibition of $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptors by etomidate and carboetomidate. Data points represent mean $\pm$ sd from at least 5 measurements at each drug concentration. Lines through data represent fits to a logistic function (Eq. 1 in Methods). Etomidate data (open circles) were fitted with $IC_{50} = 160 \mu M$ and Hill slope = 0.60. Carboetomidate data (solid triangles) were fitted with $IC_{50} = 13 \mu M$ and Hill slope = 0.87. Insets show examples of current sweeps recorded in single oocytes. Drug application is shown in the bars above each set of sweeps. Individual current sweeps are labeled with drug concentration in micromolar.