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Glycine Receptor Antagonism

Effects of ACEA-1021 on the Minimum Alveolar Concentration for Halothane in the Rat

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Background: **Glycine and glutamate binding sites are allosterically coupled at the N-methyl-D-aspartate (NMDA) receptor complex. Previous studies have shown that antagonism of glutamate at the NMDA receptor reduces the minimum alveolar concentration (MAC) for volatile anesthetics. 5-Nitro-6,7-dichloro-2,3-quinoxalinedione (ACEA-1021) is a competitive antagonist at the glycine recognition site of the NMDA receptor. The purpose of this study was to determine whether glycine receptor antagonism also reduces volatile anesthetic requirements in the rat.**

Methods: **In experiment 1, Sprague-Dawley rats were anesthetized with halothane in 50% O_2–balance N_2 and their lungs mechanically ventilated. They were randomly assigned to one of three groups according to the dose of ACEA-1021 administered (0, 20, or 40 mg/kg intravenously; n = 6). The bolus dose of ACEA-1021 was followed by a continuous intravenous infusion of vehicle or ACEA-1021 at 14 $mg \cdot kg^{-1} \cdot h^{-1}$. Halothane MAC was then determined by the tail-clamp method. In experiment 2, awake rats were randomly assigned to groups according to the same dosages of ACEA-1021 as in experiment 1. Arterial CO_2 tension and mean arterial pressure were recorded before and 5 and 30 min after the start of the infusion. The infusion was then stopped, and the time to recovery of the righting reflex was recorded.**

Results: **In experiment 1, ACEA-1021 decreased halothane MAC (mean ± SD) in a dose-dependent manner (control, 0.95 ± 0.15 vol%; ACEA-1021 20 mg/kg, 0.50 ± 0.14 vol%; ACEA-1021 40 mg/kg, 0.14 ± 0.16 vol%; $P < 0.01$). In experiment 2, arterial CO_2 tension was increased by ACEA-1021 (control, 38 ± 3 mmHg; ACEA-1021 20 mg/kg, 43 ± 3 mmHg; ACEA-1021 40 mg/kg, 48 ± 2 mmHg; $P < 0.01$). Mean arterial pressure was not affected by any dose of ACEA-1021. The righting reflex was abolished in rats receiving ACEA-1021 40 mg/kg only and recovered 30 ± 7 min after discontinuation of the infusion.**

Conclusions: **Halothane MAC reduction by glycine receptor antagonism was greater than that previously observed for antagonism of glutamate at the NMDA or AMPA receptor. In rats receiving ACEA-1021 only, minimal hemodynamic depression and moderate hypoventilation were observed. Antagonism of glycine at the NMDA receptor recognition site offers a potential mechanism of action of anesthesia. (Key words: Anesthetics, intravenous: ACEA-1021. Anesthetics, volatile: halothane. Neurotransmitters: glutamate; glycine. Receptors: glutamate; glycine.)**

THE glutamatergic N-methyl-D-aspartate (NMDA) receptor complex has been the focus of intense interest because of its putative role in the pathogenesis of neuronal cell death. Associated with the investigation has been the development of numerous specific competitive and noncompetitive glutamate NMDA receptor antagonists. These compounds (*e.g.*, dizocilpine and CGS19755) have been demonstrated to be neuroprotective against focal cerebral ischemia and to act as anticonvulsant agents.[1–4]

Concurrent with the development of these compounds has been the demonstration that substantial anesthetic properties can also be elicited, particularly when the NMDA receptor antagonists are administered in large doses.[5–7] Indeed, noncompetitive NMDA receptor antagonism is believed to be the predominant mechanism of anesthetic action for ketamine.[8,9] However, although specific NMDA receptor antagonists have allowed verification of the validity for antagonism of glutamatergic excitation as a mechanism of anesthesia, the clinical utility of these compounds has been limited by their propensity to elicit unfavorable psychotomimetic side effects.[10,11]

Alternative modes of glutamatergic antagonism are available. For example, glutamatergic coactivation of

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the AMPA receptor is required for glutamate-mediated postsynaptic depolarization. It has been shown that intravenous administration of the AMPA receptor antagonist NBQX allows substantial reduction in the minimum alveolar concentration (MAC) of halothane, accompanied by a reversible loss of consciousness in the rat.[12] Further examination of this promising mechanism of anesthesia has been limited by the availability of compounds with suitable pharmaceutical formulations and low toxicity profiles when administered in large doses.

Another mechanism for potentially inhibiting glutamatergic excitation lies in the glycine recognition site of the NMDA receptor. Glycine is required to coactivate the NMDA receptor before the associated ionophore becomes permeable.[13–16] *In vitro* electrophysiologic assays have shown that the recently synthesized compound 5-nitro-6,7-dichloro-2,3-quinoxalinedione (ACEA-1021) is a specific potent competitive antagonist at the glycine recognition site.[17] Other studies have indicated that this and other glycine antagonists are potentially neuroprotective in the context of focal cerebral ischemia and possess anticonvulsant properties.[18,19] In contrast to glutamate NMDA receptor antagonists, glycine receptor antagonists appear to be devoid of psychotomimetic properties.[20]

The purpose of this investigation was to determine whether competitive antagonism of glycine at the strychnine-insensitive NMDA receptor complex exhibits an anesthetic profile consistent with potential clinical utility of this anesthetic mechanism of action.

Materials and Methods

These studies were approved by the University of Iowa Animal Care and Use Committee.

Experiment 1

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), 13 or 14 weeks old, were allowed free access to food and water until the time of the experiment. Animals were weighed and then anesthetized with 3–4% halothane in 50% O_2–balance N_2. After orotracheal intubation, the lungs were mechanically ventilated (Rodent Ventilator 683, Harvard, South Natick, MA), to maintain arterial CO_2 tension (Pa_{CO_2}) between 35 and 45 mmHg. Halothane concentration was then adjusted to 1.3% in 50% O_2–balance N_2. Right femoral arterial and venous catheters were placed by surgical incision after local skin infiltration with 1% lidocaine. The arterial catheter was used to continuously measure mean arterial pressure (MAP) and to provide blood samples for analysis. The venous catheter was connected to a calibrated infusion pump (Apparatus Syringe infusion pump 22, Harvard) for fluid and drug administration. Rectal temperature was regulated by servomechanism at 37.0°C by surface heating or cooling. After preparation, a 20-min stabilization interval was allowed, and the inspired gas mixture changed to 1.3% halothane in 70% N_2–balance O_2. Inspired anesthetic agent concentration was continuously monitored with an (5330 Agent Monitor, Ohmeda, Louisville, CO) calibrated according to manufacturer recommendations.

Animals were assigned randomly to one of three groups (n = 6 per group) according to the dose of ACEA-1021 (ACEA Pharmaceuticals, Irvine, CA). ACEA-1021 was prepared as a 0.3% solution dissolved in D5W (5% dextrose in water) with 1% Tween 20 (Fisher Scientific, Fair Lawn, NJ) and 1.38 g/100 ml Tris(hydroxymethyl)aminomethane (Sigma, St. Louis, MO).

- control: An intravenous bolus (over a 10-min period) of vehicle, followed by an intravenous infusion of vehicle at 4.7 $ml \cdot kg^{-1} \cdot h^{-1}$
- $ACEA_1$: ACEA-1021 bolus 20 mg/kg intravenously; infusion rate 14 $mg \cdot kg^{-1} \cdot h^{-1}$ (4.7 $ml \cdot kg^{-1} \cdot h^{-1}$)
- $ACEA_2$: ACEA-1021 bolus 40 mg/kg intravenously; infusion rate 14 $mg \cdot kg^{-1} \cdot h^{-1}$ (4.7 $ml \cdot kg^{-1} \cdot h^{-1}$)

MAP was recorded before and 5 and 30 min after initial administration of the drug. In addition, 30 min after starting the infusion, 1 ml arterial blood was withdrawn for determination of plasma ACEA-1021 concentration. Analysis was performed at ACEA Pharmaceuticals by individuals blinded to group assignment. Plasma samples were vortex mixed with an equal volume of 20% trichloroacetic acid diluted in water (Fisher Scientific, St. Louis, MO) to precipitate plasma protein bound drug. The mixture was centrifuged for 5 min at 12,000 g (Microspin 12s, Sorvall Instruments, du Pont, Wilmington, DE). The supernatant was passed through a 0.45-μm nylon filter (Acrodisc, Gelman, MI). Ten microliters of sample was injected into a reverse-phase high-performance liquid chromatography column (c18) (Vydac, Hesperia, CA). The absorbance was monitored by an ultraviolet detector at 217 nm. Before analysis of the sample, a standard curve that covered the expected range of sample concentrations was gen-

erated using standards with known concentrations of ACEA-1021.

The MAC for halothane was determined starting with an end-tidal concentration of 1.3%. The painful stimulus was a rubbershod 25-cm hemostat clamped to the proximal 2 cm of the tail.[21] The clamp was applied for 60 s, during which time the hemostat was continually rotated across its long axis to simulate a wagging motion of the tail. The clamp was applied to a different site on the tail for each MAC determination. A sustained movement of any of the four extremities occurring during application of the hemostat was considered a positive response.

In the absence of movement, the inspired halothane concentration was reduced by approximately 20% (*e.g.*, to 1.1%). After 20 min of ventilation at the new inspired halothane concentration, MAP, and end-tidal halothane concentration were recorded. The stimulus was then repeated. If the animal failed to respond, the inspired halothane concentration was decreased again by 20% and the sequence was repeated until purposeful movement was observed. The value of the lowest end-tidal concentration at which the rat did not move and that end-tidal value at which movement was observed were averaged and recorded.

End-tidal halothane concentrations were determined as follows. A catheter (polyethylene tubing PE-50, Intramedic, Clay Adams, Parsippany, NJ) was permanently positioned at the distal tip of the endotracheal tube. Small aliquots ($\approx$1 ml for each ventilatory cycle) of expiratory gas were withdrawn into a glass syringe for a total of 35 ml. The aspirated gas was then introduced into a 5330 Agent Monitor (Ohmeda) for analysis.

Experiment 2

Rats were weighed and given pentobarbital (50 mg/kg intraperitoneally). Catheters (polyethylene tubing PE-50, Intramedic) were placed in the right femoral artery and vein by surgical incision and filled with heparin-containing saline. The catheters were capped, tunneled subcutaneously and exteriorized at the nape of the neck. The wounds were closed with suture and infiltrated with 0.125% bupivacaine. The animal was then allowed to awaken and recover for 24 h.

Each rat, breathing room air, was then placed in a Plexiglas cylindrical restrainer (Kent Scientific, Litchfield, CT). The arterial catheter allowed continuous recording of MAP and sampling of blood for analysis of arterial blood gases and *p*H (*p*H–blood gas analyzer IL1306, Instrumentation Laboratory, Lexington, MA). The venous catheter was used for drug infusion. The animals were assigned randomly to one of three groups (n = 6 per group). Vehicle and drug infusion regimens for the three groups (control, $ACEA_1$, and $ACEA_2$) were identical to those described above in experiment 1.

After a 15-min interval for acclimation to the restraining apparatus, baseline MAP, arterial O_2 tension (Pa_{O_2}), Pa_{CO_2}, and pH_a were determined. The designated bolus and infusion regimen was then initiated. To reflect the temporal events occurring in experiment 1, respective intravenous infusions were continued for 30 min. At 30 min after onset of infusion, MAP, Pa_{O_2}, Pa_{CO_2}, and pH_a determinations were repeated. The infusion was then discontinued. The animal was removed from the restraining apparatus and placed on its side in a cage and observed. The interval required for spontaneous recovery of the righting reflex was recorded.

To test whether ACEA-1021 decreased MAC, increased time to recovery of righting reflex, increased Pa_{CO_2} (experiment 2), or decreased MAP (experiment 2), we used one-sided linear correlation coefficients between doses (milligrams per kilogram) and responses.[22] We did not include the 0-mg/kg dose in the righting reflex analysis because the response was the same at 20 mg/kg (*i.e.*, no loss of righting reflex). Statistical power to detect a decrease in MAP, as the dose of ACEA-1021 was increased from 0 to 40 mg/kg, was calculated post hoc, using a one-tailed *t* test with α = 0.05. Values are reported as mean ± standard deviation.

Results

Experiment 1

Physiologic data are presented in table 1. Arterial *p*H, Pa_{CO_2}, Pa_{O_2}, and rectal temperature were similar between groups at time of MAC determination. MAP values were least in those rats receiving vehicle only.

ACEA-1021 decreased halothane MAC in a dose-dependent manner (control, 0.95 ± 0.15 vol%; $ACEA_1$, 0.50 ± 0.14 vol%; $ACEA_2$, 0.14 ± 0.16 vol%; $r = -0.92$, $P < 0.01$) (fig. 1). As expected, plasma concentrations of ACEA-1021 increased with larger doses of the drug (control, 0 μg/ml; $ACEA_1$, 72 ± 5 μg/ml; $ACEA_2$, 84 ± 10 μg/ml).

Experiment 2

Physiologic values are presented in table 2. Baseline values for MAP, pH_a, Pa_{CO_2}, Pa_{O_2}, and body weight were similar between groups. At completion of the bolus

Table 1. Physiologic Values for Experiment 1

	Control (n = 6)	$ACEA_1$ (n = 6)	$ACEA_2$ (n = 6)
Baseline values (pre-MAC determination)			
Body weight (g)	309 ± 19	304 ± 17	325 ± 29
MAP (mmHg)	91 ± 10	93 ± 15	107 ± 11
Values at MAC determination			
MAP (mmHg)	85 ± 16	120 ± 12	138 ± 11
Arterial *p*H	7.43 ± .06	7.44 ± .04	7.48 ± .03
Pa_{CO_2} (mmHg)	39 ± 2	39 ± 2	39 ± 2
Pa_{O_2} (mmHg)	153 ± 19	140 ± 9	147 ± 10
Rectal temperature (°C)	36.9 ± 0.1	37.0 ± 0.1	36.9 ± 0.1
Plasma ACEA-1021 (μg/ml)	0	72 ± 5	84 ± 10

Values are mean ± SD.

Control = vehicle only; $ACEA_1$ = ACEA-1021 bolus = 20 mg/kg iv, infusion rate = 14 $mg \cdot kg^{-1} \cdot h^{-1}$; $ACEA_2$ = ACEA-1021 bolus = 40 mg/kg iv, infusion rate = 14 $mg \cdot kg^{-1} \cdot h^{-1}$.

dose of ACEA-1021 or vehicle, there were no differences between groups for MAP. Thirty min after onset of infusion, MAP remained similar between the control, $ACEA_1$, and $ACEA_2$ groups ($r = -0.08$, $P = 0.38$). We had 80% power to detect a decrease in MAP of 14 mmHg. In contrast, Pa_{CO_2} increased in a dose-dependent fashion with administration of ACEA-1021 ($r = 0.96$, $P < 0.01$). Nevertheless, there was no decrease in the Pa_{O_2}.

After 30 min of ACEA-1021 (or vehicle) administration, rats in the control and $ACEA_1$ groups showed no evidence of sedation and had no impairment of the righting reflex. In contrast, all rats in the $ACEA_2$ group were deeply sedated and had a loss of the righting reflex ($P < 0.01$). Time to recovery of righting reflex after discontinuation of ACEA-1021 in these animals was 30 ± 7 min.

Discussion

The administration of the glycine-receptor antagonist ACEA-1021 resulted in a dose-dependent reduction of halothane MAC in the rat. Mean maximal reduction in MAC was 85%. Of greater interest was the observation that in five of six animals receiving the largest dose of ACEA-1021 MAC was essentially zero. In these animals, halothane was purposefully left discontinued for as long as 1 h (with continued ACEA-1021 infusion), yet no response to noxious stimuli was observed. Cohort rats, undergoing an identical infusion regimen (without halothane anesthesia), were deeply sedated but remained hemodynamically stable, although spontaneous ventilation was moderately depressed.

The magnitude of MAC reduction by glycine receptor antagonism was substantially greater than has been previously reported for compounds implicated in the antagonism of glutamatergic neurotransmission. Aside from ketamine, the original work examining effects of glutamate antagonists on volatile anesthetic requirements was that of Scheller *et al.*[5] In their experiment the noncompetitive NMDA receptor antagonist dizocilpine reduced isoflurane MAC by 65% in the rabbit albeit no attempt was made to define a ceiling effect for the drug. Other work has demonstrated that neuroprotective doses of a variety of NMDA receptor antagonists (dizocilpine, CGS19755, and CPPene) reduce isoflurane MAC by approximately 50%.[6] Using larger

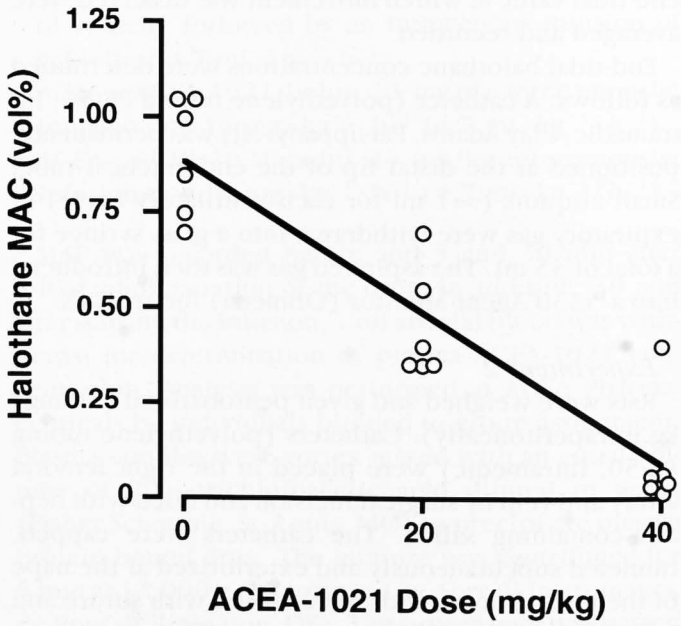

Fig. 1. Minimum alveolar concentration (MAC) for halothane as a function of the dose of the competitive glycine receptor antagonist 5-nitro-6,7-dichloro-2,3-quinoxalinedione (ACEA-1021). Each point depicts values for an individual rat. $ACEA_1$ = ACEA-1021 bolus 20 mg/kg intravenously, infusion 14 $mg \cdot kg^{-1} \cdot h^{-1}$; $ACEA_2$ = ACEA-1021 bolus 40 mg/kg intravenously, infusion 14 $mg \cdot kg^{-1} \cdot h^{-1}$. Mean ± SD MAC for halothane: control group, 0.95 ± 0.15 vol%; $ACEA_1$, 0.50 ± 0.14 vol%; $ACEA_2$, 0.14 ± 0.16 vol%. Pearson correlation coefficient, $r = -0.92$; $t = 9.31$; $P = 3 \times 10^{-8}$.

§ Perkins W, Morrow D: A dose dependent reduction in halothane M.A.C. in rats with a competitive N-methyl-D-aspartate (NMDA) receptor antagonist (abstract). Anesth Analg 74:S234, 1992.

Table 2. Physiologic Values for Experiment 2

	Control (n = 6)	$ACEA_1$ (n = 6)	$ACEA_2$ (n = 6)
Body weight (g)	304 ± 26	295 ± 23	312 ± 17
Baseline			
MAP (mmHg)	117 ± 7	124 ± 9	121 ± 4
pH_a	7.47 ± .01	7.46 ± .04	7.47 ± .03
Pa_{CO_2} (mmHg)	38 ± 2	39 ± 2	38 ± 3
Pa_{O_2} (mmHg)	85 ± 3	86 ± 3	84 ± 1
10 min after onset of ACEA-1021 (or vehicle) administration			
MAP (mmHg)	121 ± 9	124 ± 7	121 ± 7
30 min after onset of ACEA-1021 (or vehicle) administration			
MAP (mmHg)	122 ± 11	122 ± 7	121 ± 10
pH_a	7.51 ± .01	7.46 ± .02	7.44 ± .03
Pa_{CO_2} (mmHg)	38 ± 3	43 ± 3	48 ± 2
Pa_{O_2} (mmHg)	87 ± 3	94 ± 2	96 ± 6
Rectal temperature (°C)	37.0 ± 0.3	37.1 ± 0.4	37.2 ± 0.4
Plasma ACEA-1021 (μg/ml)	0	44 ± 10	78 ± 9
TTR (min)	0 ± 0	1 ± 1	30 ± 7

Values are mean ± SD.

Control = vehicle only; $ACEA_1$ = ACEA-1021 bolus = 20 mg/kg iv, infusion rate = 14 $mg \cdot kg^{-1} \cdot h^{-1}$; $ACEA_2$ = ACEA-1021 bolus = 40 mg/kg iv, infusion rate = 14 $mg \cdot kg^{-1} \cdot h^{-1}$; TTR = time to righting reflex.

doses of CPPene in the rat, Perkins and Morrow reported a ceiling effect for the drug as a 78% reduction in halothane MAC.§ Previous work in our laboratory has focused on antagonism of glutamate at the AMPA receptor with NBQX; the maximal reduction in halothane MAC that could be obtained was approximately 60%.[12] Toxic side effects precluded administration of larger doses of the drug. Finally, presynaptic inhibition of glutamate release with the anticonvulsant agent riluzole again caused an approximately 50% reduction in halothane MAC in the rat.[7]

Thus, the results from this experiment are encouraging that a reversible deep anesthetic state can be achieved solely through antagonism of glycine at the NMDA receptor complex. Several considerations, however, remain to be resolved. First, current pharmaceutical development of glycine receptor antagonists is at a stage where bioavailability remains a problem and the magnitude of specificity for the glycine receptor by these agents is largely unpublished. It is likely that weak coantagonism of glutamate at the kainate (inhibition constant K_i = 92 μM) or AMPA (K_i = 20 μM) receptor (as opposed to the strychnine-insensitive glycine receptor, K_i = 0.007 μM) is a property of these compounds when administered in large doses (although there is no affinity for ACEA-1021 and either the strychnine-sensitive glycine receptor or γ-aminobutyric acid A or γ-aminobutyric acid B receptors).[23] At least three reports have implicated a synergism between compounds that are targeted to antagonize glutamate at the NMDA or AMPA receptor,[24–26] leaving open the possibility that the magnitude of effect we observed was attributable to a combined antagonism of different receptor types.

Furthermore, an important question has been raised with respect to what MAC actually represents. It is unsettling to conclude that a drug provides anesthesia simply on the basis of a MAC reduction study, given the work of Rampil *et al.*[27–29] In a series of ablation experiments, the possible anatomic locus of MAC has been moved progressively caudal in the neuroaxis, indicating that MAC represents a spinal phenomenon.[27–29] The spinal cord is richly endowed with glutamatergic neurotransmitters and to some extent, the results observed in this experiment may be directly attributable to spinal effects of the glycine antagonist on neurotransmission. However, this investigation has identified a potent sedative effect for ACEA-1021 suggesting a central role, at least in part, for the anesthetic effects of glycine receptor antagonists.

Finally, we cannot be sure of the effect of ACEA-1021 on the efficiency of the neuromuscular junction. There is little obvious reason to expect this class of compounds would exert neuromuscular blockade, which might have precluded movement to the noxious stimuli. No attempt was made to assess the integrity of the neuromuscular junction in this experiment. However, given the relatively modest compromise of ventilation observed (Pa_{CO_2} 48 ± 2 mmHg and Pa_{O_2} 96 ± 6 mmHg) in rats spontaneously breathing room air at the largest dose of ACEA-1021 it seems unlikely that substantial neuromuscular blockade was present.

In conclusion, competitive antagonism at the glycine receptor by intravenous ACEA-1021 was effective in reducing the halothane MAC in a dose-dependent fashion. There was no evidence of hemodynamic compromise at doses sufficient to reduce halothane MAC by as much as 85%. In contrast, mild hypercapnia was observed without hypoxemia with the largest doses administered. Given the known specificity of ACEA-1021, the data show that competitive glycine antagonism at the strychnine-insensitive NMDA receptor complex modulates anesthetic potency as measured by the reduction in halothane MAC. The results implicate gly-

cine antagonism at the strychnine-insensitive glycine recognition site of the NMDA receptor complex as a site for potentiation of general anesthetic action. It is hoped that this work will provide a basis for the development of agents that in addition to having anesthetic potential will be neuroprotective.

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