

Additive Interaction of the Cannabinoid Receptor 1 Agonist Arachidonyl-2-chloroethylamide with Etomidate in a Sedation Model in Mice

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Background: Both propofol and volatile anesthetics have been reported to interact with the endocannabinoid system. The purpose of this study was to evaluate the effect of selective agonists for cannabinoid receptor types 1 and 2 on etomidate-induced sedation.

Methods: A controlled, blinded, experimental study was performed in 20 mice that received intraperitoneal injections of etomidate, the cannabinoid₁ receptor agonist arachidonyl-2-chloroethylamide (ACEA), the cannabinoid₂ receptor agonist JWH 133 alone, and both ACEA and JWH 133 combined with etomidate. The cannabinoid₁ receptor antagonist AM 251 and the cannabinoid₂ receptor antagonist AM 630 were administered 10 min before the delivery of ACEA and JWH 133, respectively. Each drug combination was applied to 6–8 mice of these 20 study animals. Sedation was monitored by a Rota-Rod (Ugo Basile, Comerio, Italy). Isobolographic analysis was used for evaluation of pharmacologic interaction.

Results: Single drug administration of etomidate and ACEA produced dose- and time-dependent decreased time on the Rota-Rod ($P < 0.05$). No sedative effect was seen after JWH 133. Etomidate-induced sedation was significantly increased and prolonged with ACEA ($P < 0.05$), but not with JWH 133. Isobolographic analysis revealed an additive interaction between ACEA and etomidate that was antagonized by the cannabinoid₁ receptor antagonist AM 251. The cannabinoid₁ receptor antagonist had no effect on etomidate alone.

Conclusions: Etomidate-induced sedation was increased and prolonged by activation of the cannabinoid₁ receptor, but not of the cannabinoid₂ receptor, in mice. However, this interaction was only additive.

ENDOCANNABINOIDS have been demonstrated to play an important role in the physiologic control of sleep, sedation, anxiety, pain processing, and emesis, suggesting a possible role as adjuvants during anesthesia.¹ The endocannabinoid system includes two identified cannabinoid receptors: type 1, which mainly exists in the central nervous system, and type 2, which is absent from the brain but is enriched in peripheral neuronal and immune tissues.² It has recently been proposed that the

anesthetic drug propofol induces an increase in the brain content of the endocannabinoid anandamide and that this may contribute to the sedative effects of propofol.³ Furthermore, volatile anesthetic-evoked sleep duration has been reported to be prolonged by different exogenously administered cannabinoids.⁴

Etomidate (*R*-(+)-ethyl-1-(1-phenylethyl)-1H-imidazole-5-carboxylate) is a widely used potent hypnotic drug whose major advantage has been described as hemodynamic stability. This pharmacologic profile renders etomidate particularly suitable for induction of anesthesia in critically ill patients and patients with cardiovascular disease.⁵ The anesthetic effect is thought to be mediated primarily through an action on γ -aminobutyric acid receptors.⁶ In addition, interactions of etomidate with α_2 adrenoreceptors⁷ and the nitric oxide metabolism⁸ have been suggested.

To elucidate the role of cannabinoid receptors in the anesthetic action of etomidate, we studied the interaction of etomidate with selective agonists and antagonists for cannabinoid_{1/2} receptors *in vivo* in mice. We hypothesized that the activation of the cannabinoid₁ receptor increases etomidate-induced sedation, but not activation of the cannabinoid₂ receptor.

Materials and Methods

Animals

This project was approved by the Animal Investigation Committee of the University Schleswig-Holstein, Campus Kiel, Germany, and the animals were managed in accordance with institutional guidelines. This was a controlled, blinded, randomized, experimental study in 20 mice (129S2/SVHsd) of either sex, weighing 25–35 g. Mice were housed 4 animals per cage and maintained on a 12-h light–dark cycle with free access to water and food. All experiments were conducted between 08:00 and 18:00 h.

A total of 20 mice were used in this study. Each following drug combination was applied to 6–8 mice of these 20 study animals. Thus, each animal was repeatedly exposed to different drug combinations. To avoid any interference with drug remnants from the previous regimen, a washout period of at least 20 days was chosen.

Drugs

Lipid emulsion (Lipofundin® 20%; B. Braun, Melsungen, Germany) was used as the solvent for etomidate (Etomi-

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date®-Lipuro; B. Braun) and as an inactive control. Arachidonyl-2-chloroethylamide (ACEA) and JWH 133 (Tocris Bioscience, Ellisville, MO) are cannabinoid₁ and cannabinoid₂ receptor agonists with 1,400-fold⁹ and 200-fold¹⁰ selectivity for binding to the cannabinoid₁ and cannabinoid₂ receptor *in vitro*, respectively. AM 251 and AM 630 (Tocris Bioscience) are cannabinoid₁ and cannabinoid₂ receptor antagonists with 306-fold¹¹ and 165-fold¹² selectivity for binding to the cannabinoid₁ and cannabinoid₂ receptors *in vitro*, respectively. ACEA, JWH 133, AM 251, and AM 630 were dissolved in ethanol, Cremophor (Sigma-Chemie, Deisenhofen, Germany), and saline in a 1:1:18 ratio. Solvents were also used as vehicle control for cannabinoid_{1/2} receptor agonists and antagonists, respectively. All drugs were administered intraperitoneally in a volume of 10 ml/kg body weight, and animals were weighed on the day of the experiment for calculation.

Sedation

Sedation was determined by placing mice on a rotating wheel (Rota-Rod; Ugo Basile, Comerio, Italy), and measuring the duration of time they remained on the rod as described previously.⁷ Mice were initially trained until they could stay on the Rota-Rod for at least 60 s at a speed of 28 revolutions per minute. Time on the Rota-Rod was recorded 1, 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 75, and 90 min after drug administration. The observer was blinded with respect to the drugs applied.

Agonists and Etomidate

For evaluation of the single drug dose response of the sedative action of the cannabinoid₁ receptor agonist (ACEA, 2.5, 3, 4, 5, 6, 8, 10, and 15 mg/kg), cannabinoid₂ receptor agonist (JWH 133, 2.5, 5, 10, and 15 mg/kg), and hypnotic drug (etomidate, 0.5, 1, 2, 4, 5, 6, 8, and 10 mg/kg), each agent was intraperitoneally administered. ED₅₀ values of ACEA and etomidate were separately calculated representing the effective dose that produced a reduction in time on the Rota-Rod to an average of 30 s in the six to eight mice tested. Injection time of each single drug experiment was defined as $t = 0$.

Role of Cannabinoid Receptor Subtypes

To further determine whether the effects of ACEA and JWH 133 were mediated through certain subtypes of cannabinoid receptors, the cannabinoid₁ receptor antagonist (AM 251, 5 mg/kg) and cannabinoid₂ receptor antagonist (AM 630, 5 mg/kg) were administered 10 min before the delivery of ACEA (ED₅₀) and JWH 133 (5 mg/kg), respectively. Then, we investigated the drug combination of 5 mg/kg AM 251, ED₅₀ ACEA, and ED₅₀ etomidate. Furthermore, we evaluated the role of the endocannabinoid system in etomidate-induced sedation. Therefore, we examined the effect of the cannabinoid_{1/2} receptor antagonists (AM 251, 5 mg/kg; and

AM 630, 5 mg/kg) combined with both lipid emulsion (0.2 mg/kg) and etomidate (ED₅₀) on Rota-Rod performance, respectively.

Drug Interactions

An isobolographic analysis¹³ was used to determine the nature of pharmacologic interaction between ACEA and etomidate. This method is based on comparisons of doses that are determined to be equieffective. First, each ED₅₀ value was determined from the single drug dose-response curves. Next, ACEA was coadministered with etomidate or 0.2 mg/kg lipid emulsion ($t = 0$) in a fixed 1:1 ratio of their respective agonist ED₅₀ values (0.1, 0.25, 0.33, 0.4, 0.5, 0.6, and 1.0). From the dose-response curve of the combined drugs, the ED₅₀ value of the mixture was calculated. The isobologram was constructed by plotting the ED₅₀ values of the single agents on the x- and y-axes, respectively. The theoretical additive dose combination was calculated.

Statistical Analysis

Statistics were performed using commercially available statistics software (GraphPad Prism version 4.03 for Windows; GraphPad Software, San Diego, CA). A Kolmogorov-Smirnov test was used to test for gaussian distribution. Data were analyzed using two-way repeated-measures analysis of variance factoring for time and drug effects with *post hoc* Bonferroni correction. Data are expressed as mean \pm SEM. The dose-response lines were fitted using least-squares linear regression and ED₅₀. Drug combinations were analyzed for additive interactions using a "fixed ratio design" isobologram whereby combinations of two drugs in known ratios were administered as fractions of their respective ED₅₀, as outlined above.¹³ The isobologram consists of an additivity line that connects the ED₅₀ of ACEA on the vertical axis to the ED₅₀ of etomidate on the horizontal axis. The theoretical dose required for a purely additive interaction ($Z_{\text{add}} = (f)ED_{50, \text{drug A}} + (1 - f)ED_{50, \text{drug B}}$, where f is the fraction of drug A used) was calculated and compared *via* an unpaired Student t test to the actual dose (Z_{mix} , determined from the ED₅₀ of the combination dose-response curve) required to achieve the same effect experimentally. Statistical significance was considered at a two-sided P value of less than 0.05.

Results

Single drug administration of etomidate and ACEA to conscious mice produced dose- and time-dependent decreased time on the Rota-Rod (figs. 1A and B; $P < 0.05$). JWH 133 in different dosages from 2.5 to 15 mg/kg did not affect ability to remain on the Rota-Rod. Rota-Rod values at the time points at which the greatest sedative responses were observed for each respective drug were

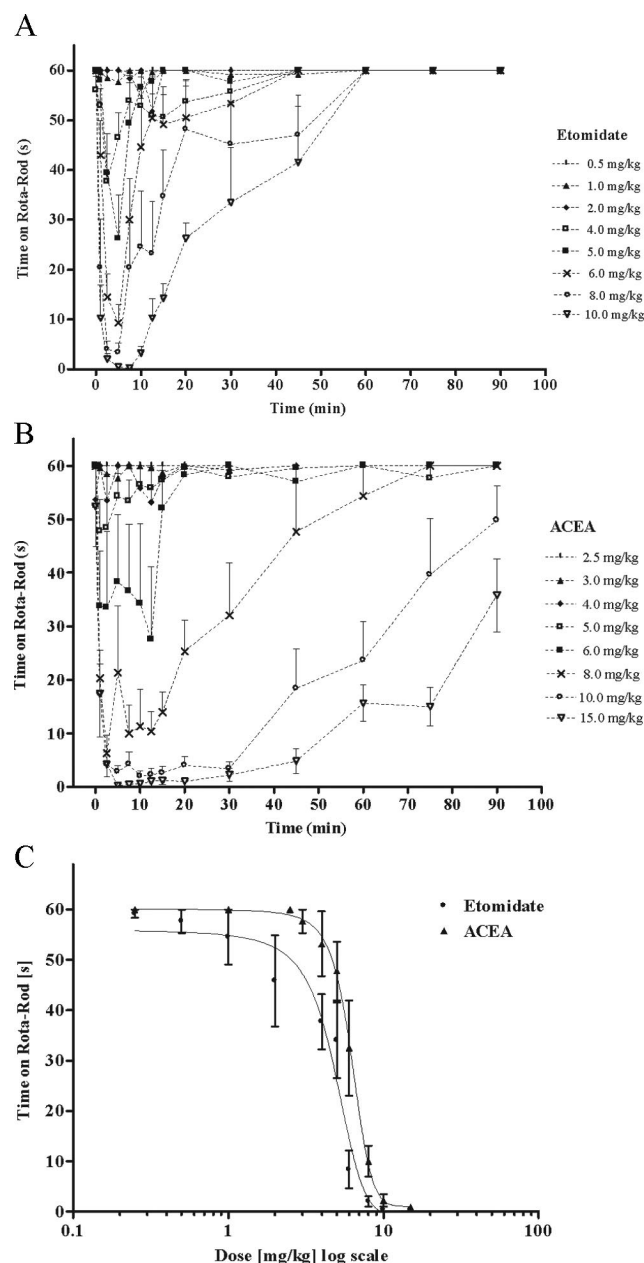


Fig. 1. Dose- and time-dependent sedative effects of single intraperitoneal bolus administration of etomidate (0.5–10 mg/kg; **A**) and arachidonyl-2-chloroethylamide (ACEA; 2.5–15 mg/kg; **B**) in the Rota-Rod test (Rota-Rod; Ugo Basile, Comerio, Italy) in mice. Time on the Rota-Rod (in seconds) is shown from 0 min (bolus injection) to 90 min after administration. Each *data point* provided represents the mean of six to eight animals, including the respective SEM indicated by the *whiskers*. Rota-Rod values at the time point at which the greatest sedative responses were observed for each respective drug were used to plot the dose–response curves shown in **C**. Etomidate and ACEA produced a dose-dependent decrease of time on the Rota-Rod ($P < 0.05$). Comparison of curve fits revealed that a sigmoidal dose–response model with variable slope fits best for etomidate (goodness of fit, $R^2 = 0.7034$) and ACEA ($R^2 = 0.7779$).

used to plot the agonist log dose–response curves displayed in figure 1C. The mean ED_{50} values (\pm SEM) of etomidate and ACEA were 4.84 (\pm 0.35) and 6.23 (\pm 0.40) mg/kg, respectively. Comparison of curve fits

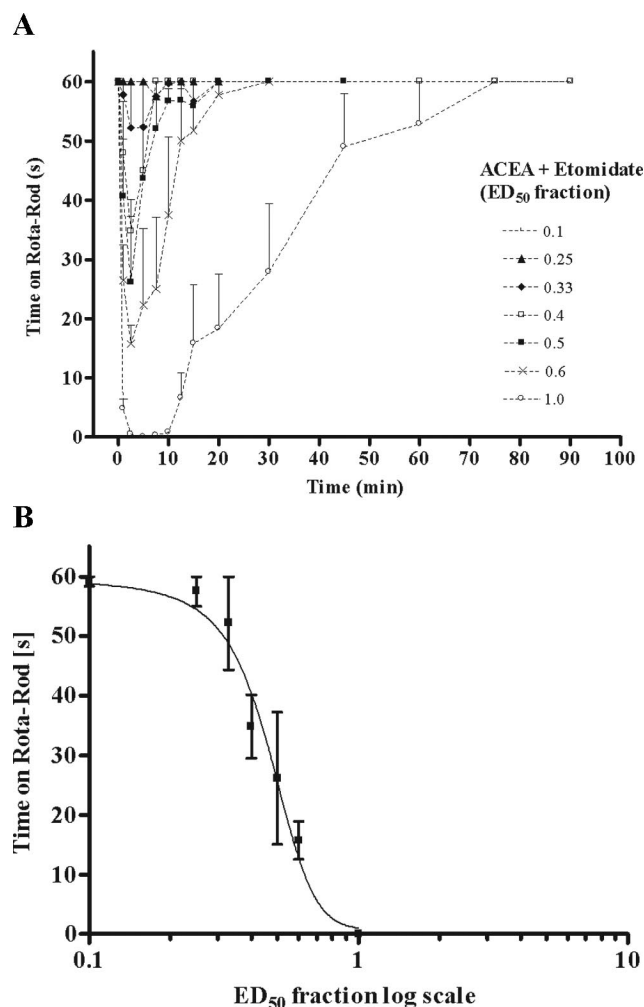


Fig. 2. Dose- and time-dependent sedative effects of paired intraperitoneal combinations of arachidonyl-2-chloroethylamide (ACEA) and etomidate in the Rota-Rod test (Rota-Rod; Ugo Basile, Comerio, Italy) in mice (**A**). The combinations were of equal fractions (0.1, 0.25, 0.33, 0.4, 0.5, 0.6, and 1.0) of each paired drug's respective ED_{50} value coadministered in a fixed 1:1 ratio of the ED_{50} of ACEA: ED_{50} of etomidate. Time on the Rota-Rod (in seconds) is shown from 0 min (bolus injection) to 90 min after administration. Each *data point* provided represents the mean of six to eight animals, including the respective SEM indicated by the *whiskers*. Rota-Rod values at the time point at which the greatest sedative responses were observed for each respective combination were used to plot the dose–response curve shown in **B**. Paired combinations of etomidate and ACEA produced a dose-dependent decrease of time on the Rota-Rod ($P < 0.05$). Comparison of curve fits revealed that a sigmoidal dose–response model with variable slope fits best for the combination of ACEA and etomidate ($R^2 = 0.7467$).

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Dose- and time-dependent sedative effects of the cannabinoid₁ receptor agonist ACEA combined with etomidate are shown in figure 2A, and combinations were of equal fractions (0.1, 0.25, 0.33, 0.4, 0.5, 0.6, and 1.0) of each paired drug's respective ED_{50} value coadministered in a fixed 1:1 ratio of the ED_{50} of ACEA: ED_{50} of etomidate. Rota-Rod values at the time points at which the

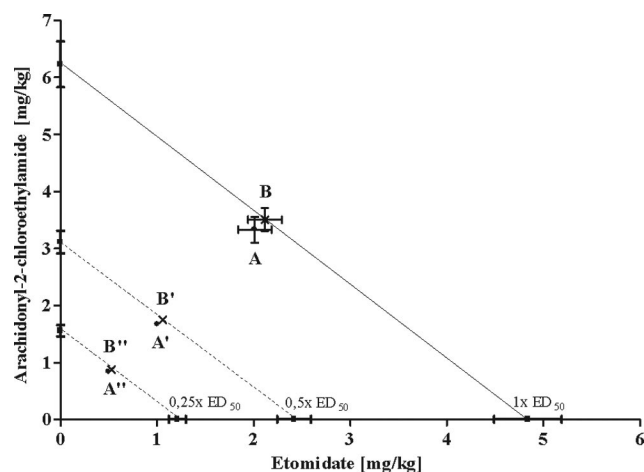


Fig. 3. Isobologram for the interaction between arachidonyl-2-chloroethylamide and etomidate in the Rota-Rod test (Rota-Rod; Ugo Basile, Comerio, Italy). The ED_{50} values for etomidate and arachidonyl-2-chloroethylamide are plotted on the x - and y -axes, respectively, and the **thick lines** represent the SEM of the ED_{50} . The **straight line** connecting each ED_{50} value is the theoretical additive line, and **point B** on this line is the theoretical additive ED_{50} . In addition, respective $0.5 \times ED_{50}$ and $0.25 \times ED_{50}$ values, lines of additivity (**dashed**), and experimental (A' , A'') and theoretical ED_{50} points (B' , B'') are presented. The experimental ED_{50} points (A' , A'') did not differ significantly from the theoretical ED_{50} points (B' , B''), respectively, indicating an additive interaction.

greatest sedative responses were observed for each respective combination were used to plot the dose-response curve shown in figure 2B. Paired combinations of ACEA and etomidate produced a dose-dependent decrease of time on the Rota-Rod ($P < 0.05$). Dose fraction (an arbitrary value) ED_{50} values were determined and converted to absolute dose values for isobolographic analysis. The ED_{50} value (\pm SEM) of the fixed-ratio combination ACEA and etomidate was $0.47 (\pm 0.04)$. Comparison of curve fits revealed that a sigmoidal dose-response model with variable slope provided the best fit for the combination of ACEA and etomidate ($R^2 = 0.7467$). The cannabinoid₂ receptor agonist JWH 133 combined with ED_{50} etomidate did not change time on the Rota-Rod compared with ED_{50} etomidate alone.

Accordingly, isobolographic analysis revealed an additive interaction between intraperitoneal ACEA and etomidate. The experimental ED_{50} value (A) did not significantly differ from the theoretical ED_{50} value (B) ($P = 0.5787$; fig. 3). Experimentally obtained (Z_{mix}) and theoretical (Z_{add}) additive doses of ED_{50} , ED_{30} , ED_{25} , and ED_{20} are presented in table 1.

The cannabinoid₁ receptor antagonist AM 251 reversed the sedative effect of single drug administration of ED_{50} ACEA ($P < 0.01$), and the sedative component of ACEA when ED_{50} ACEA was combined with ED_{50} etomidate ($P < 0.01$; fig. 4A). In contrast, the cannabinoid_{1/2} receptor antagonists AM 251 and AM 630 combined with ED_{50} etomidate did not significantly differ from ED_{50} etomidate alone. Further, AM 251 and AM 630 combined

Table 1. Calculation of the Experimentally Obtained and Theoretical Additive Doses

Dose Level	Z_{mix} , Mean (95% CI)	Z_{add} , Mean (95% CI)	P Value
ED_{50}	5.225 (4.726–5.724)	5.449 (4.922–5.976)	0.5787
ED_{30}	4.313 (3.760–4.865)	4.514 (3.943–5.086)	0.6398
ED_{25}	4.042 (3.417–4.667)	4.234 (3.637–4.830)	0.6715
ED_{20}	3.732 (3.006–4.458)	3.911 (3.283–4.538)	0.7107

The experimental dose (Z_{mix}) has been derived from log linear regression analysis of the dose-response curve of arachidonyl-2-chloroethylamide combined with etomidate. The theoretical dose (Z_{add}) required for a purely additive interaction was calculated as $Z_{add} = (f)ED_{x, \text{drug A}} + (1 - f)ED_{x, \text{drug B}}$, where f is the fraction of drug A used, and ED_x is the respective ED_{50} , ED_{30} , ED_{25} , and ED_{20} . Statistical comparison of Z_{mix} and Z_{add} was performed with an unpaired t test. At none of the dose levels applied was any statistically significant difference obtained, indicating a simple additive interaction between arachidonyl-2-chloroethylamide and etomidate.

CI = confidence interval.

with 0.2 mg/kg lipid emulsion did not affect baseline Rota-Rod performance (fig. 4B).

Discussion

Etomidate is widely used for induction of anesthesia, particularly in critically ill patients, because of its beneficial properties, including rapid, predictable onset of action, cardiovascular stability, and short half-life.⁵ In agreement with previous experimental studies,^{14,15} intraperitoneal injection of etomidate reduced time on the Rota-Rod, an index of the sedative action of general anesthetics in mice, in a dose-dependent manner.

Main findings of our experimental study in mice are as follows. First, etomidate and the cannabinoid₁ receptor agonist ACEA alone reduced time on the Rota-Rod in a dose-dependent manner, indicating increased sedation, whereas the cannabinoid₂ receptor agonist JWH 133 had no effect, irrespective of the dosage used. Second, etomidate-induced sedation was significantly increased and prolonged with ACEA, but not with JWH 133. However, isobolographic analysis revealed that this interaction is based on simple additivity. Third, the anesthetic action of etomidate is not mediated *via* cannabinoid receptors.

With regard to natural cannabinoids, their analgesic and sedative properties have historically been used during surgical procedures more than three centuries ago.¹⁶ In our experimental study, the synthetic cannabinoid₁ receptor agonist ACEA altered the Rota-Rod performance by decreasing time on the Rota-Rod in a dose-dependent manner, whereas the cannabinoid₂ receptor agonist JWH 133 had no effect. Cannabinoid₁ receptors are located throughout the central nervous system, including the neocortex, hippocampus, basal ganglia, and brainstem,¹⁷ regions that have been associated with sedation.¹⁸ In this respect, sleep duration of volatile anesthetics such as halothane or isoflurane has been reported to be prolonged when combined with both nonselective and selective cannabinoid₁ receptor agonists.⁴ Delta-9-

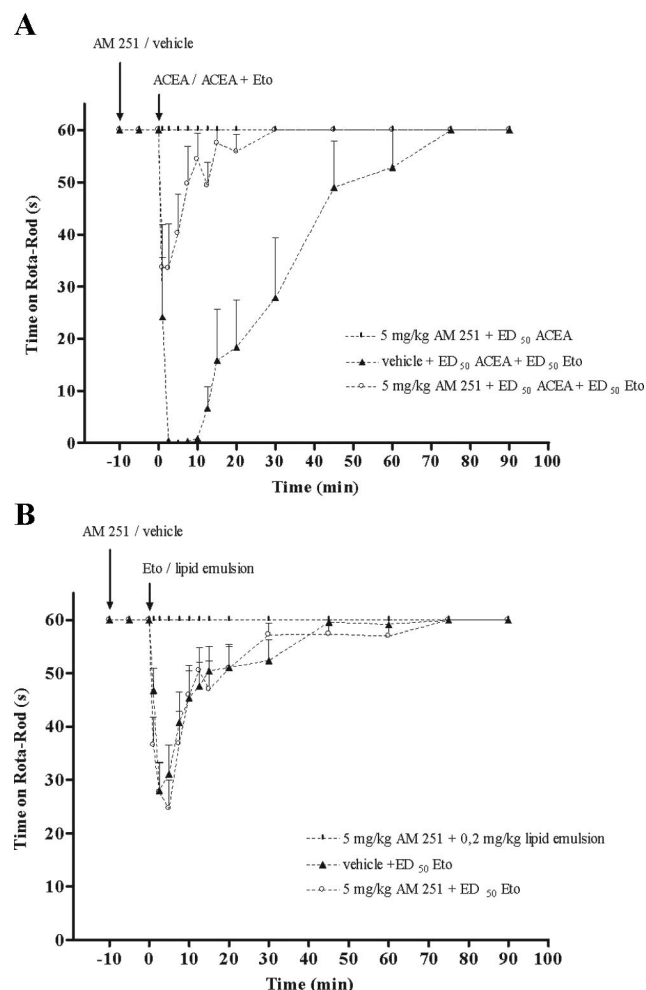


Fig. 4. (A) Dose- and time-dependent sedative effects of intraperitoneal bolus administration of (1) 5 mg/kg AM 251 combined with ED₅₀ arachidonyl-2-chloroethylamide (ACEA), (2) ED₅₀ ACEA combined with ED₅₀ etomidate (Eto), and (3) 5 mg/kg AM 251 combined with ED₅₀ ACEA and ED₅₀ etomidate. The cannabinoid₁ receptor antagonist AM 251 reversed the sedative component of ACEA both when ACEA was administered alone and in combination with etomidate. (B) Dose- and time-dependent sedative effects of (1) 5 mg/kg AM 251 combined with 0.2 mg/kg lipid emulsion, (2) vehicle combined with ED₅₀ etomidate, and (3) 5 mg/kg AM 251 combined with ED₅₀ etomidate. Thus, the cannabinoid₁ receptor antagonist had no effect on etomidate-induced sedation. Time on the Rota-Rod (in seconds) (Rota-Rod; Ugo Basile, Comerio, Italy) is shown from -10 min to 90 min after administration. AM 251 and vehicle control were administered at $t = -10$ min, and ACEA, etomidate, and lipid emulsion at $t = 0$. Each data point provided represents the mean of six to eight animals, including the respective SEM indicated by the whiskers.

Tetrahydrocannabinol enhanced thiopental-induced loss of righting reflex, too.¹⁹ In addition, propofol-evoked loss of righting reflex was increased by coadministration of a nonselective cannabinoid₁ receptor agonist.³ These authors have further suggested that propofol induces an inhibition of the anandamide-degrading enzyme, the fatty acid amide hydrolase that leads to elevated concentration of anandamide, an endogenous nonselective cannabinoid_{1/2} receptor ligand, which in turn may contribute to the sedative effects of propofol. More recently, even a

reduced anandamide concentration has been reported after etomidate administration in patients, suggesting counteracting effects of etomidate and fatty acid amide hydrolase.²⁰

The current study indicates that activation of the cannabinoid₁ receptor by ACEA increased and prolonged significantly etomidate-induced sedation, suggesting a potentially anesthetic-sparing effect. Furthermore, isobolographic analysis of this study revealed that our results for the combination of ACEA and etomidate represent a simple additive interaction, suggesting that activation of both cannabinoid receptors and γ -aminobutyric acid receptors cause sedation by independent mechanisms or sites of action. However, the fact that lower doses of sedative drugs may be administered in combination to cause effective sedation may have potential clinical benefit. Additive drug combinations may enhance the pharmacodynamic safety margin because the lower clinical dose requirements for each agent will minimize drug-specific adverse effects.²¹ In addition, as etomidate is not used for repetitive administration and long-term sedation because of its detrimental effect on adrenal function,²² enhanced and prolonged sedative effects after a single etomidate injection might be advantageous under special circumstances.

With respect to an appropriate effect size for the difference between the actually measured additive dose, Z_{mix} , and the theoretical one, Z_{add} , we considered a difference of 10% or greater between the observed and expected absolute dose in mg/kg of etomidate or ACEA to be clinically meaningful. At none of the four different fractional ED₅₀ levels did we obtain any such difference. Hence, not only did the Student t test give a nonsignificant result, but also the mean data differed by less than the clinically relevant effect size. Therefore, it can reasonably be concluded that the interaction is simply additive.

Furthermore, a pharmacokinetic alteration of the endocannabinoid system by etomidate³ is unlikely because an inhibition of fatty acid amide hydrolase by etomidate has not yet been demonstrated, and ACEA metabolism is independent of fatty acid amide hydrolase.²³ Moreover, an interaction between ACEA and the lipid solvent contained in the etomidate emulsion also seems highly improbable, because the combination of both drugs did not affect Rota-Rod performance. In addition, although etomidate is indeed known as an inhibitor of cytochrome P450 3A4, ACEA has, to the best of our knowledge, not been reported as a substrate, inhibitor, or inducer of any CYP isoenzyme including CYP3A4. Hence, CYP-mediated drug-drug interactions are also unlikely. However, it remains speculative whether other interactions between ACEA and etomidate, especially given by the intraperitoneal route, may have influenced the results obtained.

In terms of pretreatment of mice with the cannabinoid₁ receptor antagonist AM 251 that did not significantly change etomidate-induced sedation, sedative properties of

etomidate may not depend on activation of cannabinoid_{1/2} receptors by endocannabinoids *per se*, whereas an endogenous cannabinoid tone mediated by cannabinoid₁ receptors has been suggested to contribute to sedative-hypnotic effects of propofol.³ To further determine whether the effects of cannabinoids were mediated through certain subtypes of cannabinoid receptors, the cannabinoid₁ receptor antagonist AM 251 and cannabinoid₂ receptor antagonist AM 630 were administered 10 min before the delivery of ACEA and JWH 133, respectively. Therefore, AM 251 reversed the sedative component of ACEA when ACEA was administered both alone and in combination with etomidate. In contrast to cannabinoid₁ receptor activation, the cannabinoid₂ receptor agonist JWH 133 did not affect etomidate-induced Rota-Rod performance. This difference is not astonishing, because cannabinoid₂ receptors have been demonstrated to be predominantly expressed in peripheral neuronal tissue and in the immune system,² and single cannabinoid₂ receptor activation did not induce impairment in motor coordination in our study.

Several limitations to this study should be noted. First, the use of the intraperitoneal route enables hepatic metabolism, and we did not determine serum concentration or brain content of the drugs applied and their active metabolites. Second, we did not perform any ligand-binding studies to elucidate a direct activation of cannabinoid₁ receptors by etomidate. Third, effects of drugs given throughout the study on systemic hemodynamic and respiratory variables were not evaluated. Further, both the timing and the dose of the cannabinoid₁ receptor antagonist used may be responsible for the negative effect on etomidate-induced sedation. However, 5 mg/kg AM 251 reversed the sedative effect of single drug administration of ACEA completely. Therefore, the dose range of AM 251 used in our study may provide sufficient antagonistic properties at the cannabinoid₁ receptor when combined with etomidate. With respect to effect site concentrations, in the clinical context, dosing of anesthetic drugs is usually accomplished irrespective of plasma concentrations. Hence, our results are particularly meaningful because they translate from dose to response as opposed to concentration to response. Finally, data from animals should be extrapolated to humans with caution.

In conclusion, activation of the cannabinoid₁ receptor, but not of the cannabinoid₂ receptor, resulted in increased and prolonged etomidate-evoked sedation based on an additive interaction. Therefore, these data suggest

that selective cannabinoid₁ receptor agonists could be novel targets for anesthetic drug development.

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