Ketamine Action in the *In Vitro* Cortical Slice Is Mitigated by Potassium Channel Blockade

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ABSTRACT

Background: Ketamine is a general anesthetic thought to act by antagonizing *N*-methyl-D-aspartate receptors. However, ketamine acts on multiple channels, many of which are potential targets—including hyperpolarization-activated cyclic nucleotide-gated and potassium channels. In this study we tested the hypothesis that potassium leak channels contribute to the anesthetic action of ketamine.

Methods: Adult mouse cortical slices (400 μm) were exposed to no-magnesium artificial cerebrospinal fluid to generate seizure-like event activity. The reduction in seizure-like event frequency after exposure to ketamine (n = 14) was quantified as a signature of anesthetic effect. Pharmacologic manipulation of hyperpolarization-activated cyclic nucleotide-gated and potassium channels using ZD7288 (n = 11), cesium chloride (n = 10), barium chloride (n = 10), low-potassium (1.5 mM) artificial cerebrospinal fluid (n = 10), and urethane (n = 7) were investigated.

Results: Ketamine reduced the frequency of seizure-like events (mean [SD], –62 [22]%, $P < 0.0001$). Selective hyperpolarization-activated cyclic nucleotide-gated channel block with ZD7288 did not significantly alter the potency of ketamine to inhibit seizure-like event activity. The inhibition of seizure-like event frequency by ketamine was fully antagonized by the potassium channel blockers cesium chloride and barium chloride (8 [26]% and 39 [58%] increase, respectively, $P < 0.0001$, compared to ketamine control) and was facilitated by the potassium leak channel opener urethane (–93 [8]%, $P = 0.002$ compared to ketamine control) and low potassium artificial cerebrospinal fluid (–86 [11]%, $P = 0.004$ compared to ketamine control).

Conclusions: The results of this study show that mechanisms additional to hyperpolarization-activated cyclic nucleotide-gated channel block are likely to explain the anesthetic action of ketamine and suggest facilitatory action at two-pore potassium leak channels. **(ANESTHESIOLOGY 2018; 128:1167-74)**

THE molecular mechanism of ketamine has long been presumed to reside in its well-described *N*-methyl-D-aspartate (NMDA) receptor–blocking action.[1] However, the centrality of NMDA receptors to the action of ketamine can be challenged on multiple levels. For example, neither the potent NMDA antagonist MK-801[2] nor genetic ablation of the NR2A NMDA-receptor subunit[3] causes anesthesia in mice. While a modest reduction in the potency of ketamine is seen in NR2A knockouts,[3,4] this can be attributed to NMDA-independent compensatory effects[4]—perhaps related to circadian rhythms.[3] While NMDA receptors cannot be completely disregarded, additional targets are required to fully explain how ketamine causes anesthesia.[5]

Like most other general anesthetics, ketamine is a promiscuous drug with actions at multiple levels within the nervous system—from ion channels, to neuromodulatory systems, to genetic expression pathways.[5–7] Any one (or many) of these could contribute to ketamine anesthesia. Two prime candidates within the central nervous system are hyperpolarization-activated cyclic nucleotide-gated and potassium channels. Ketamine stereoselectively blocks hyperpolarization-activated cyclic nucleotide-gated–1 channels, causing membrane hyperpolarization[8]—while hyperpolarization-activated cyclic nucleotide-gated–1 channel knockout mice are ketamine-resistant.[8] These findings are mirrored by the general anesthetic propofol and together make a strong case for hyperpolarization-activated cyclic nucleotide-gated–1 channel involvement. Comparatively little is known about the action of ketamine on potassium channels. It blocks

What We Already Know about This Topic

- The conventional view is that ketamine exerts its anesthetic action by antagonism of the *N*-methyl-D-aspartate (NMDA) receptor. That other potent and specific NMDA receptor antagonists do not produce anesthesia suggests that ketamine may well modulate other non-NMDA receptor targets.

What This Article Tells Us That Is New

- In addition to the previously demonstrated inhibition of hyperpolarization-activated cyclic nucleotide-gated channels, ketamine has a facilitatory action at two-pore potassium channels. The available data suggest that ketamine produces anesthesia by multiple mechanisms that include *N*-methyl-D-aspartate receptor antagonism, hyperpolarization-activated cyclic nucleotide-gated channel antagonism, and facilitation of two-pore potassium channels.

Submitted for publication July 19, 2017. Accepted for publication January 29, 2018. From the Anaesthesia Department, Waikato District Health Board, Hamilton, New Zealand (L.J.V., S.K.); the School of Science and Engineering, University of Waikato, Hamilton, New Zealand (V.E.); and the University of Auckland Waikato Clinical School, Hamilton, New Zealand (J.W.S.).

<zdoi;10.1097/ALN.0000000000002147>

neuronal voltage-sensitive potassium channels but at concentrations that are probably not clinically relevant.[9] A more likely target is the diverse family of two-pore potassium channels.[10] Collectively, these so-called "leak" channels regulate the resting membrane potential—and are clinically relevant targets for other anesthetics such as volatiles and anesthetic gases.[11,12] The effect of ketamine on two-pore potassium channels has not been thoroughly investigated,[13] with the exception of the acid-sensitive TWIK-related-acid-sensitive potassium-3 (TASK-3) channels, which appear to be largely ketamine-resistant.[14] Apart from the weak inward rectifying adenosine triphosphate–sensitive potassium channels,[15] neuronal potassium inward rectifiers have not been investigated for ketamine actions.

In this study, we sought to further clarify the action of ketamine by investigating hyperpolarization-activated cyclic nucleotide-gated and potassium channels, utilizing an established *in vitro* model for investigating anesthesia drug effects.[16] The cortical slice no-magnesium seizure-like event model is a well-established tool for investigating seizure mechanisms. We have shown that this model also has utility for investigating mechanisms of anesthetic action—in that anesthetic inhibition of seizure-like event activity in the slice is a pathognomonic signature of *in vivo* behavioral hypnosis.[16] We hypothesized that pharmacologic manipulation of potassium channels would alter the suppressant effect of ketamine on seizure-like event activity—consistent with a role for potassium leak channels in its mechanism of action.

Materials and Methods

The tissue recovery methods in this study were approved by the Animal Ethics Committee at the University of Waikato (Hamilton, New Zealand).

Preparation of Slices

We investigated coronal brain slices from 21 wild-type mice with the genetic background C57. The animals were of both sexes and their age ranged from 2 to 6 months old. They were kept in a temperature-controlled room with a 12-h day/night cycle and had unlimited access to food and water.

After being anesthetized with carbon dioxide, the mice were decapitated and the brain rapidly dissected into ice-cold "normal" artificial cerebrospinal fluid buffered with either HEPES or bicarbonate. Either carbogen (95% oxygen, 5% carbon dioxide) or 95% oxygen (Perfecto2 oxygen concentrator, Invacare, New Zealand) was used for artificial cerebrospinal fluid oxygenation. HEPES normal artificial cerebrospinal fluid was composed of 130 mM sodium chloride, 2.5 mM potassium chloride, 1 mM magnesium chloride, 2 mM calcium chloride, 10 mM HEPES, and 20 mM D-glucose (in double-distilled water). Bicarbonate normal artificial cerebrospinal fluid was composed of 125 mM sodium chloride, 2.5 mM potassium chloride, 1 mM magnesium chloride, 2 mM calcium chloride, 1.25 mM sodium dihydrogen phosphate, 26 mM sodium bicarbonate, and 10 mM D-glucose (in double-distilled water). All artificial cerebrospinal fluid constituents were from Sigma (USA), except HEPES (ITW Reagents, Spain) and sodium chloride (EMSURE, Denmark).

After removal of the most posterior and anterior coronal sections with a razor blade, the remaining part of the brain (between Bregma 1 and –5, approximately) was glued onto a metallic plate and sectioned in ice-cold normal artificial cerebrospinal fluid into 400-μm-thick coronal slices (Vibratome, Campden Instruments Ltd., United Kingdom). The slices were transferred to no-magnesium artificial cerebrospinal fluid buffered with either HEPES or bicarbonate for at least 1 hour of recovery at room temperature. Either carbogen (95% oxygen, 5% carbon dioxide) or 95% oxygen (Perfecto2 oxygen concentrator) were used for no-magnesium artificial cerebrospinal fluid oxygenation. HEPES no-magnesium artificial cerebrospinal fluid was composed of 130 mM sodium chloride, 5 mM potassium chloride, 2 mM calcium chloride, 10 mM HEPES, and 20 mM D-glucose. Bicarbonate no-magnesium artificial cerebrospinal fluid was composed of 130 mM sodium chloride, 5 mM potassium chloride, 2 mM calcium chloride, 1.25 mM sodium dihydrogen phosphate, 26 mM sodium bicarbonate, and 10 mM D-glucose. The pH of the HEPES-buffered solutions was adjusted to 7.4 with 10 M sodium hydroxide.

After recovery, the slices were transferred to a submersion-style perfusion bath (Kerr Scientific Instruments, New Zealand) for recording at room temperature. Before being placed in the bath, the two hemispheres of the slice were separated by cutting the corpus callosum, making it possible to record independent activity from each hemisphere. Thus, each slice provided two recordings for analysis. The bath was perfused continuously with carbogenated/oxygenated no-magnesium artificial cerebrospinal fluid by gravity-feed at a rate of 5 ml/min.

Electrical Recording

For recording spontaneous local field potential activity, either 50 μm Teflon-coated tungsten (California Fine Wire Company, USA) or silver/silver chloride (GoodFellow Ltd., United Kingdom) electrodes were positioned in the cortex of each hemisphere. The signal was amplified (×250, Kerr Scientific Instruments, New Zealand; or ×1,000, A-M Systems, USA) and digitally filtered (100 Hz low-pass and 1 Hz high-pass) before analog-to-digital conversion (PowerLab, ADInstruments, Australia; or CED, United Kingdom) and storage on computer for later analysis. To limit electrical noise interference, the experiments were performed in a Faraday shielded room.

Drug Preparation and Delivery

All drugs were added directly to carbogenated no-magnesium artificial cerebrospinal fluid from stock solutions. Ketamine (Ketalar, Hospiro Australia Pty. Ltd., Australia) was delivered at 17 μM for all experiments; propofol (Provive,

Claris, India) 56 μM; ZD7288 (HelloBio, United Kingdom) 10 μM; cesium chloride (Sigma, USA), 2 mM and 200 μM; urethane (Sigma, USA), 20 mM and 40 mM; and barium chloride (Sigma, USA), 200 μM. The ZD7288 solution was made fresh each day. The artificial cerebrospinal fluid solutions were stored at 4°C for no longer than 2 weeks.

Experimental Protocols

No formal statistical power analysis was undertaken to determine sample size. The group sizes were estimated from previous experiments in which a sample size of 5 to 10 has been shown to be sufficient to detect statistically significant drug effects on seizure-like event frequency. The main experimental protocol is illustrated in figure 1. Briefly, each experiment was preceded by a baseline recording period in which stable seizure-like event activity was recorded for at least 10 min in no-magnesium artificial cerebrospinal fluid (5 mM potassium). Thereafter, no-magnesium artificial cerebrospinal fluid containing ZD7288 (n = 11, from five animals); cesium chloride, 2 mM and 200 μM (n = 10 and n = 7 respectively, from three animals); low potassium, 1.5 mM (n = 10, from two animals); urethane 20 mM and 40 mM (n = 7 and n = 5, respectively, from one animal), or barium chloride (n = 10, from one animal) was perfused. The drugs were perfused for 30 min and low-potassium for 20 min. Ketamine was then added and perfused for 15 min, before washout with drug-free no-magnesium artificial cerebrospinal fluid until stable seizure-like event activity returned. One recording from the cesium chloride 200 μM group was excluded from the analysis because seizure-like event activity did not return after drug washout. Because the sequence of drug delivery was integral to the rationale of the study, no randomization was employed. The experimenter was not blinded to drug conditions.

The concentration-response for barium chloride at 2 μM (n = 5), 20 μM (n = 5), 200 μM (n = 4), and 2,000 μM (n = 5) was quantified in a separate set of experiments. For these experiments, ketamine and barium chloride were perfused concurrently for 15 min immediately after the baseline period, effectively eliminating the "Q2" period in figure 1. Drug delivery was followed by washout with drug-free no-magnesium artificial cerebrospinal fluid.

Two sets of control experiments were undertaken. First, ketamine controls (n = 14, from four animals) were exposed to ketamine only for 15 min after the baseline recording period. Second, the low-potassium experiments were repeated with propofol. Propofol controls (n = 8, two animals) were exposed to propofol for 30 min with standard artificial cerebrospinal fluid potassium concentrations (5 mM); a second group (n = 9, two animals) was exposed to propofol for 30 min with reduced artificial cerebrospinal fluid potassium concentrations (1.5 mM).

Because some of these experiments were also testing a developmental ketamine analog (results not reported), on some occasions (n = 6) the analog was perfused before ketamine in the protocol. In these cases, the analog was washed out and stable seizure-like event activity recovered before beginning the ketamine perfusion.

Statistical Analysis

The recorded data were exported to MatLab (The Mathworks Inc., USA) for analyzing. Background noise and artefacts were removed by visual inspection. Seizure-like event frequency was quantified across the duration of each recording using custom-written MatLab scripts. Select time periods were chosen for averaging and statistical comparison (fig. 1), as follows:

(1) No-magnesium baseline: 5 min before the first intervention being administrated ("Q1" in fig. 1)
(2) Intervention (ZD7288, cesium chloride, low potassium, urethane, and barium chloride): 5 min before start of ketamine perfusion ("Q2" in fig. 1)
(3) Ketamine + intervention: 5 min from the start of washout ("Q3" in fig. 1)

Statistical analysis was performed in Graphpad (Graphpad Software Inc., USA). Unless otherwise stated in the text, data are presented as mean (SD). The data were tested for normality using the Kolmogorov-Smirnov test, followed by the appropriate pairwise (paired *t* test or Wilcoxon) or unpaired (unpaired *t* test or Mann-Whitney) test. Normally distributed data are presented as mean (SD), and nonnormally distributed data are presented as median (interquartile range). For family-wise multiple comparisons, a P value < 0.0167 was considered statistically significant, with Bonferroni correction for multiple (3) tests. Otherwise, $P < 0.05$ was considered statistically significant.

Results

Baseline Seizure-like Event Activity

All slices generated spontaneous seizure-like event activity when perfused with no-magnesium artificial

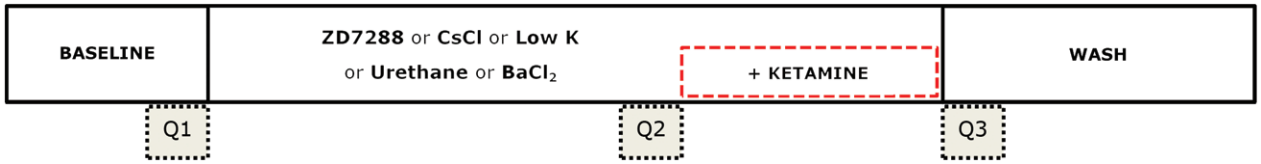

Fig. 1. Schematic showing the protocol sequence for the main interventional part of the study. The *gray boxes* labeled *Q* represent the time periods during which seizure-like event frequency was quantified for statistical comparison. Note that the maximum ketamine effect tended to occur in the period immediately after terminating drug perfusion, hence the alignment of *Q3* with the beginning of the Wash period. $BaCl_2$ = barium chloride; CsCl = cesium chloride; Low K = low potassium (1.5 mM); Wash = perfusion with drug-free artificial cerebrospinal fluid.

Table 1. Effect of Test Agents on the Frequency of Seizure-like Event Activity

	Baseline Seizure-like Event Frequency (events/min)	Test Seizure-like Event Frequency (events/min)
Ketamine	4.0 (0.9)	1.5 (0.9)*
ZD7288	3.3 (3.1)	4.0 (2.0)
Cesium (2 mM)	4.6 (1.1)	12.6 (4.4)†
Cesium (200 μM)	2.1 (1.5)	2.5 (1.8)
Barium	2.5 (0.9)	6.3 (1.7)‡
Low potassium (1.5 mM)	2.9 (1.2)	2.5 (0.8)
Urethane (20 mM)	2.3 (0.9)	2.8 (1.4)
Urethane (40 mM)	2.3 (0.7)	0.4 (0.4)§

The data reflect Q1 and Q2 in Figure 1. Data for ZD7288 and barium are listed as median (interquartile range). All other data are listed as mean (SD). *P < 0.0001, paired t test. †P < 0.0005, paired t test. ‡P < 0.005, Wilcoxon test. §P < 0.005, paired t test.

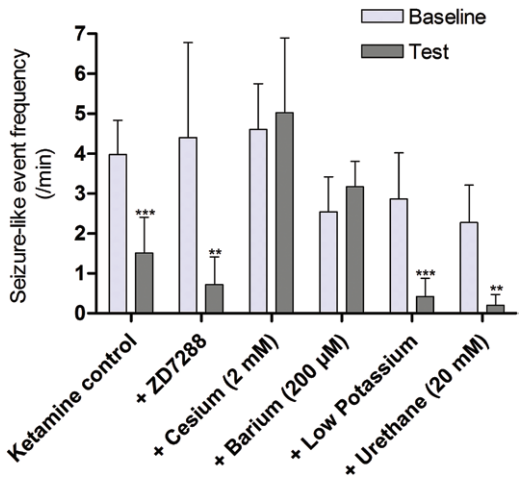

Fig. 2. *Bar chart* showing the effect of each intervention on the ability of ketamine to reduce seizure-like event frequency. The two potassium channel–enhancing interventions increased the ketamine effect, while the two potassium channel–blocking interventions reduced the ketamine effect. Data are mean (SD). The data reflect *Q1* and *Q3* in Figure 1. *P < 0.05, compared to ketamine, unpaired t test. **P < 0.005, compared to ketamine, unpaired t test. ***P < 0.0001, compared to ketamine, unpaired t test.

cerebrospinal fluid. There was no difference in baseline seizure-like event frequency in slices prepared in HEPES *versus* bicarbonate-buffered artificial cerebrospinal fluid (results not shown). Seizure-like events are seen as positive or negative field potential "spikes" that deviate strongly from the background "noise," with or without an oscillatory tail of varying length. The frequency, length, and amplitude of the seizure-like events vary somewhat between slices. For the purpose of this investigation, we have focused on seizure-like event frequency effects—that is, the interval between discrete events—distinct from the frequency of the oscillatory component within an individual event. Seizure-like event frequency effects were of paramount importance for this analysis because it is specifically this characteristic of seizure-like event activity that is consistently and robustly modulated by anesthetic drugs.[16,17] In particular, the potency of an anesthetic to cause behavioral anesthesia *in vivo* relates directly to the capacity of that drug to reduce the frequency of seizure-like event activity in the cortical slice.[16]

Ketamine Effects

In keeping with previous investigations,[16,17] ketamine under control conditions effected a strong reduction in seizure-like event frequency in all slices (62 [22]% reduction, P < 0.0001; table 1). Perfusing the ketamine test analog before did not significantly alter the magnitude of the ketamine inhibitory effect.

Effect of Hyperpolarization-activated Cyclic Nucleotide-gated Channel Blockade with ZD7288 and Cesium Chloride

No change in seizure-like event frequency *per se* was seen during exposure to the hyperpolarization-activated cyclic nucleotide-gated channel antagonist ZD7288 (10 μM; table 1). We noted that a higher concentration of ZD7288 (100 μM) reduced seizure-like event frequency strongly (data not shown).

ZD7288 did not alter the ketamine inhibition of seizure-like event activity compared to controls. The ketamine-induced reduction in seizure-like event frequency during ZD7288 exposure was –82 (18)% (P = 0.02 compared to ketamine control), considered not significant with Bonferroni correction (fig. 2). Cesium chloride was tested as a second, somewhat less specific, hyperpolarization-activated cyclic nucleotide-gated channel blocker. At 2 mM it caused a quite dramatic increase in seizure-like event frequency in all slices tested, with a mean increase of 188 (127)% from baseline (P = 0.0003; table 1). The ketamine inhibitory effect was prevented in the presence of 2 mM cesium chloride (fig. 2; P < 0.0001 compared to ketamine control). Cesium chloride at 200 μM had no effect on seizure-like event frequency (table 1) or on the ketamine inhibition of seizure-like event activity (–54 [48]% reduction compared to –62 [22]% reduction with ketamine alone, P = 0.63). Together these findings suggest that hyperpolarization-activated cyclic nucleotide-gated channel blockade is not the only contributor to the anesthetic action of ketamine.

Effect of Potassium Channel Modulation

In addition to its well-documented effect on hyperpolarization-activated cyclic nucleotide-gated channels,[18] higher (mM) concentrations of cesium chloride are known to block both inwardly rectifying and leak potassium channels.[13,18,19] Because the hyperpolarization-activated cyclic nucleotide-gated channel–specific drug ZD7288 was without effect on ketamine, we concluded that the attenuation of ketamine inhibition of seizure-like event activity by cesium was likely to be a potassium channel effect—with leak channel blockade specifically implicated. Direct enhancement

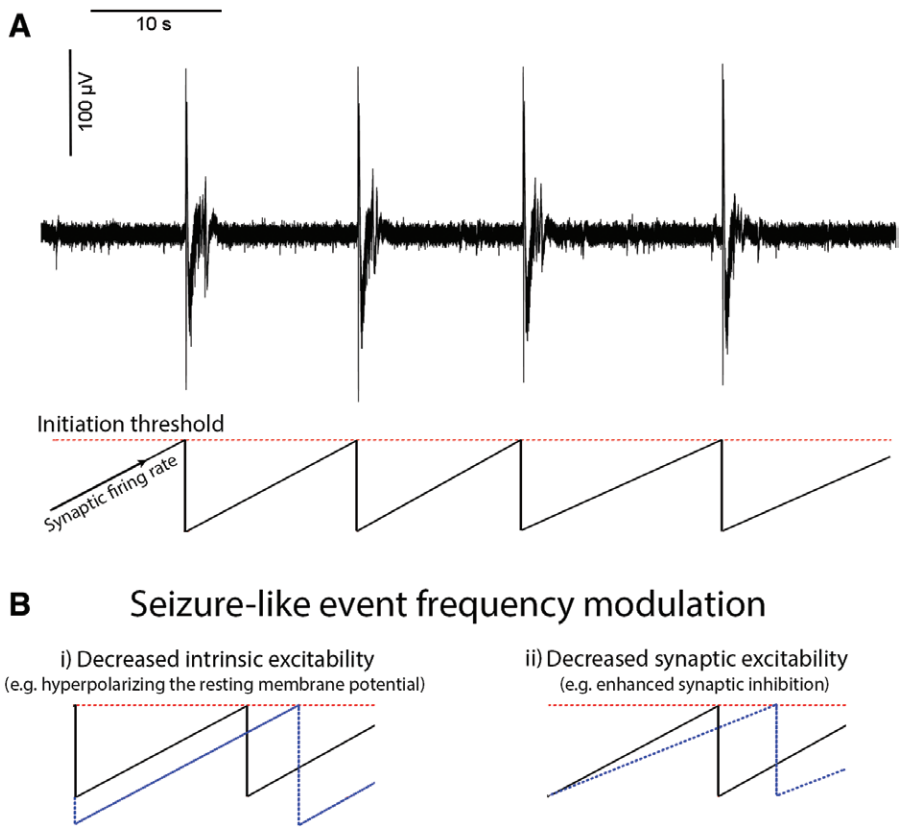

Fig. 3. Schematic showing (*A*) an example seizure-like event recording and (*B*) proposed mechanisms for modulation of event frequency by alterations in intrinsic neuronal excitability and synaptic excitability. The initiation of epileptiform population events (and hence, event frequency) in brain slices is dependent upon an interevent "recovery" process that drives synaptic firing toward an excitability threshold.[35] The timing of this recovery process is variable and depends upon both intrinsic and synaptic excitability.[35] Anesthetic regulation of seizure-like event frequency exemplifies both processes. A reduction in intrinsic excitability causing hyperpolarization of the resting membrane potential decreases seizure-like event frequency by shifting away from the firing rate threshold (in the case of potassium leak channel agonists such as urethane[21]). A reduction in synaptic excitability decreases seizure-like event frequency by reducing the rate of return to the firing rate threshold (in the case of γ-aminobutyric acid agonists such as etomidate[36]).

of seizure-like event activity by cesium is consistent with this interpretation. We examined this hypothesis further by investigating the effect on ketamine of manipulating potassium channel activity with low artificial cerebrospinal fluid potassium, potassium channel opening with urethane, and blockade with barium chloride.

We postulated that if ketamine opens potassium leak channels, enhancing potassium leak currents should heighten the ketamine suppression of seizure-like event activity. We reduced artificial cerebrospinal fluid potassium from 5 mM to 1.5 mM, which, according to the potassium Nernst potential and the Goldman equation, would have a hyperpolarizing effect by way of increased outward movement of potassium through leak channels. Decreasing artificial cerebrospinal fluid potassium to 1.5 mM on its own had no effect on seizure-like event frequency (table 1), but significantly enhanced the ketamine inhibition of seizure-like events (–86 [11]%, P = 0.004 compared to ketamine control; fig. 2). Importantly, low artificial cerebrospinal fluid potassium had no effect on seizure-like event inhibition by propofol, a general anesthetic void of any potassium channel effects.[20] With standard potassium (5 mM), propofol reduced seizure-like event frequency by –57 (30)%, compared to –67 (24)% with low potassium (1.5 mM; P = 0.44).

With enhanced potassium leak conductance implicated in the action of ketamine, we investigated the effect of pharmacologic manipulation of potassium leak channels. Urethane is a central nervous system depressant that activates barium-sensitive potassium leak channels.[21] At a concentration sufficient to significantly reduce cortical neuronal spike rates *in vitro* (greater than 10 mM[21]), we found that urethane (20 mM) had no inhibitory effect on seizure-like events (table 1), but significantly enhanced the ketamine inhibition of seizure-like event frequency (–93 [8]%, P = 0.002 compared to ketamine control; fig. 2). We note that a higher concentration of urethane (40 mM) itself strongly and reversibly depressed seizure-like event frequency (table 1), consistent with general anesthetic effects on seizure-like event activity.

Barium chloride at low concentrations (less than 200 µM) blocks potassium leak currents, with TWIK-related

Table 2. Barium Concentration-Response on the Ketamine Inhibition of Seizure-like Event Activity

	Baseline Seizure-like Event Frequency (events/min)	Test Seizure-like Event Frequency (events/min)
Ketamine	4.0 (0.9)	1.5 (0.9)*
Ketamine + barium chloride (2 μM)	1.3 (0.5)	0.7 (0.4)†
Ketamine + barium chloride (20 μM)	2.4 (1.0)	2.9 (2.6)
Ketamine + barium chloride (200 μM)	4.4 (1.3)	3.3 (1.0)
Ketamine + barium chloride (2,000 μM)	2.3 (0.9)	1.8 (0.7)

Data for ketamine + barium chloride (200 μM) are listed as median (interquartile range). All other data are listed as mean (SD).

*$P < 0.0001$, paired t test. †$P < 0.005$, paired t test.

potassium (TREK) and weakly inward rectifying potassium (TWIK)-mediated currents particularly implicated.[21–23] Significantly, barium chloride (200 μM) eliminated the ketamine suppression of seizure-like event frequency (fig. 2). In keeping with a potassium channel–blocking action, the effect of barium chloride in the absence of ketamine was similar to that of cesium chloride, with a median (interquartile range) increase in seizure-like event frequency of 138 (68)% from baseline ($P = 0.002$; table 1). A concentration-response analysis showed that antagonism of ketamine by barium had a threshold of approximately 20 μM (table 2). Together, the data points to enhancement of potassium leak conductances as an important action of ketamine.

Discussion

The aim of this study was to investigate two possible molecular targets for the general anesthetic ketamine—hyperpolarization-activated cyclic nucleotide-gated and potassium channels. The former has been implicated as an important site for ketamine hypnotic anesthetic action.[8] The hyperpolarization-activated cyclic nucleotide-gated channel blocker ZD7288[24] had no effect on seizure-like event frequency. While the mean reduction in event frequency was lower when ZD7288 and ketamine were combined, this difference was not statistically significant. Drug dosing in avascularized slices is an inexact science, and it's possible the concentration used was on the low side. When applied at 100 μM, ZD7288 strongly inhibited seizure-like events; however, we were concerned that this might be attributable to voltage-gated sodium channel blockade at this concentration.[25] It seems hyperpolarization-activated cyclic nucleotide-gated channels probably contribute to the anesthetic action of ketamine, in keeping with previous studies[8]; however, the modest effect in the slice model points to the possibility of additional mechanisms. Cesium chloride is commonly used along with ZD7288 as a hyperpolarization-activated cyclic nucleotide-gated channel blocker,[18] but with a number of off-target effects.[18,26] Pretreatment with cesium chloride enhanced seizure-like events and prevented the ketamine inhibitory effect on seizure-like event frequency—effects opposite to that expected if cesium chloride and ketamine were both blocking hyperpolarization-activated cyclic nucleotide-gated–1 channels. Because ZD7288 was without effect on seizure-like event frequency, we concluded that the cesium chloride effect was unlikely to be a hyperpolarization-activated cyclic nucleotide-gated channel action.

An explanation for enhancement of seizure-like event activity by cesium may lie in an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) agonist action. Cesium chloride causes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-dependent seizures in hippocampal slices.[26] However, this can be discounted in our study, because the cesium chloride excitatory effect was not blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (results not shown). An alternative and more likely explanation is that cesium chloride is antagonizing either leak or inward rectifying potassium channels.[13,18,19] The latter seems unlikely because urethane does not act on inward rectifying potassium channels[21]—yet enhanced ketamine in this study. On the other hand, potassium leak inhibition, causing membrane depolarization, would explain the cesium chloride–dependent excitation. Most interestingly, its attenuation of the ketamine inhibitory action implies that ketamine is acting *via* an opposing mechanism—that is, by opening potassium leak channels. The elimination of the ketamine inhibitory effect by barium chloride offers strong support for this, as does the enhancement of ketamine by urethane and low artificial cerebrospinal fluid potassium.

In this study we have interpreted the blocking effect of barium on ketamine as a "pharmacologic" antagonism—an opposing action at a common receptor. While our data strongly implicate potassium channels in the ketamine effect, we cannot completely rule out a "physiologic" antagonistic effect—an opposing action of ketamine independent of potassium channels. More specific pharmacologic blockers, genetic manipulations, and/or targeted electrophysiologic studies are required to unequivocally determine whether one or other leak channels are activated by ketamine—and the extent to which these effects contribute to its anesthetic profile.

Barium chloride blocks numerous potassium leak conductances at millimolar concentrations,[23] but at lower concentrations its effect is more targeted toward TWIK-related potassium (TREK) and weakly inward rectifying potassium (TWIK) channels.[27–29] At the 200-μM concentration used in this study, we would expect greater than 50% blockade of TWIK-related potassium (TREK) and weakly inward rectifying potassium (TWIK) channels.[27–29] TWIK-related potassium-1 (TREK-1) channels are insensitive to lower concentrations of cesium chloride,[29] consistent with our finding that 200 μM cesium chloride had no effect on seizure-like event activity. Weakly inward rectifying potassium-1 (TWIK-1) potassium channels are expressed extensively

in the central nervous system,[30] but compared to TWIK-related potassium-1 (TREK-1) (and TWIK-related acid-sensitive potassium [TASK]) channels[31,32] have not been widely implicated in anesthesia mechanisms.[33] While antagonism of TWIK-related acid-sensitive potassium (TASK) and weakly inward rectifying potassium (TWIK) channels cannot be ruled out,[23] the former at least seems unlikely because urethane does not act on TWIK-related acid-sensitive potassium (TASK) channels.[21]

The magnitude of seizure-like event frequency reduction with anesthetics correlates with *in vivo* anesthetic potency,[16] the quantification of which provides a useful tool for investigating anesthetic mechanisms. The basis of the relationship between seizure-like event frequency and anesthetic effect can be understood in terms of the processes that regulate rhythmic population bursting in brain slice preparations (fig. 3). It must be acknowledged that extrapolation to clinical outcomes with *in vitro* models such as this should be done cautiously. While we have found the correlation between *in vivo* anesthetic effect and seizure-like event inhibition to be surprisingly consistent,[16,17] the seizure-like event model closely reflects an epileptiform state, which itself is a complex activity that is not thoroughly understood. Utilizing pharmacologic agents that have various off-target effects makes it difficult to draw unequivocal conclusions around the role of potassium channels in the mechanism of ketamine action.

In this study we utilized multiple slices from relatively few animals. This increases the potential issue of the dependence of data sampled from the same animals. To address this, we quantified the within-animal and between-animal variance in both baseline seizure-like event frequency and change in seizure-like event frequency in response to test-drug perfusion (results not shown). Baseline seizure-like event frequency tended to be more similar in slices obtained from the same animal, compared to between animals. However, the magnitude of the change in seizure-like event frequency was independent of multiple observations from the same animal. Because the data analysis is based entirely on the change in seizure-like event frequency, we do not believe the results were animal-dependent.

Drug dosing in avascularized slices is not straightforward. In this study we chose a ketamine concentration of 17 μM, equivalent to 4 μg/ml. Hypnotic concentrations of ketamine fall in the range of 1 to 4 μg/ml for major procedures,[34] meaning the concentration used was at the upper end of clinical relevance. However, an avascularized slice relies on passive drug diffusion, a relatively time-intensive process. Thus, for a drug exposure period of only 15 min, the actual drug concentration in the middle (healthiest) section of the slice will be somewhat less than the drug concentration in the artificial cerebrospinal fluid. In conclusion, the results of this study show that mechanisms additional to hyperpolarization-activated cyclic nucleotide-gated channel block are likely to explain the anesthetic mechanism of ketamine and point to a facilitatory action at two-pore potassium leak channels.

Research Support

Support was provided solely from institutional and/or departmental sources.

Competing Interests

The authors declare no competing interests.

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