Published in final edited form as:

Anesthesiology. 2015 April ; 122(4): 806–820. doi:10.1097/ALN.0000000000000583.

Enhanced Effects of Isoflurane on the Long QT Syndrome 1 associated A341V Mutant

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Abstract

Background—The impact of volatile anesthetics on patients with inherited long QT syndrome (LQTS) is not well understood. This is further complicated by the different genotypes underlying LQTS. No studies have reported on the direct effects of volatile anesthetics on specific LQTS-associated mutations. We investigated the effects of isoflurane on a common LQTS-Type 1 mutation, A341V, with an unusually severe phenotype.

Methods—Whole-cell potassium currents (IKs) were recorded from HEK293 and HL-1 cells transiently expressing/co-expressing wild-type KCNQ1 (α -subunit), mutant KCNQ1, wild-type KCNE1 (β -subunit), and fusion KCNQ1+KCNE1. Current was monitored in the absence and presence of clinically relevant concentration of isoflurane (0.54 ± 0.05 mM, 1.14 vol%). Computer simulations determined the resulting impact on the cardiac action potential.

Results—Isoflurane had significantly greater inhibitory effect on A341V+KCNE1 ($62.2 \pm 3.4\%$, $n=8$), than on wild-type KCNQ1+KCNE1 ($40.7 \pm 4.5\%$; $n=9$) in transfected HEK293 cells. Under heterozygous conditions, isoflurane inhibited A341V+KCNQ1+KCNE1 by $65.2 \pm 3.0\%$ ($n=13$), and wild-type KCNQ1+KCNE1 (2:1 ratio) by $32.0 \pm 4.5\%$ ($n=11$). A341V exerted a dominant negative effect on IKs. Similar differential effects of isoflurane were also observed in experiments

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The authors declare no competing interests.

Funding Disclosure: This study was supported by National Institutes of Health (Bethesda, MD, USA) grant R01 GM-067675 (WMK) and the Medical College of Wisconsin (Milwaukee, WI, USA) Diversity Summer Health-related Research Education Program (CGT).

using the cardiac HL-1 cells. Mutations of the neighboring F340 residue significantly attenuated the effects of isoflurane, and fusion proteins revealed the modulatory effect of KCNE1. Action potential simulations revealed a stimulation-frequency dependent effect of A341V.

Conclusions—The LQTS-associated A341V mutation rendered the IKs channel more sensitive to the inhibitory effects of isoflurane compared to wild-type IKs in transfected cell lines; F340 is a key residue for anesthetic action.

Introduction

The long QT syndrome (LQTS) is a cardiac disease characterized by abnormal prolongation of the QT interval in the electrocardiogram, which can lead to syncope and sudden death.¹ LQTS can be inherited or acquired.² For inherited LQTS, mutations in thirteen different genes have been identified and categorized as LQT1 – 13.^{3–8} The penetrance of inherited LQTS was initially thought to be limited, but recent reports suggest a higher prevalence, which may be higher still when latent or concealed LQTS is factored in.^{9,10} Furthermore, drug-induced LQTS may also be a pharmacogenomic syndrome predisposed by rare genetic variants.¹¹

In the perioperative setting, there is a high risk of arrhythmias in patients with inherited arrhythmogenic syndromes such as LQTS, and the occurrences of life-threatening arrhythmias in congenital LQTS patients during general anesthesia have been reported.^{12,13} Although the impact of general anesthesia on LQTS patients have been discussed,^{14–16} anesthetic management of patients with diagnosed inherited arrhythmias or those carrying silent mutations remains a challenge. Because of the impact of volatile anesthetics on cardiac ion channels, and consequently on the QT interval, these agents can potentially exacerbate perioperative arrhythmias in patients diagnosed or suspected with inherited arrhythmias. The effects of these agents may also be dependent on the specific genotype of the underlying LQTS. No previous studies have directly investigated the actions of volatile anesthetics on any of the known LQTS-associated mutations in the cardiac ion channels.

A majority of the mutations are associated with LQT1 – 3 with underlying defects in the slowly activating delayed rectifier potassium (IKs) channel, the rapidly activating delayed rectifier potassium (IKr) channel, and the cardiac sodium channel, respectively. The prevalence for LQT1 is greater than those for LQT2 and 3.¹⁷ The acquired form of LQTS is most commonly associated with the block of the IKr channel.¹⁸ Interestingly, the documented ability of volatile anesthetics to prolong the QT interval^{19–21} is likely due to the inhibition of the IKs, rather than of the IKr, channel.^{22,23} Although volatile anesthetics have been shown to modulate various types of cardiac voltage-gated ion channels, their effects on IKs appeared to be the most significant.²²

Due to the sensitivity of IKs to volatile anesthetics, the use of these agents can potentially worsen the already compromised repolarization reserve in patients with inherited LQTS. Our goal was to investigate the effects of isoflurane on a mutant IKs associated with LQT1. IKs consists of the pore-forming α -subunit and accessory β -subunit encoded by KCNQ1 and KCNE1, respectively.^{24,25} Several mutations in KCNQ1 have been identified in LQT1²⁶ that result in either a dominant suppression of channel expression or changes in its

biophysical characteristics.^{27,28} We focused on an alanine to valine mutation at position 341 in the 6th transmembrane (S6) domain of the KCNQ1, namely A341V.^{29–32} This mutation is associated with an unusually severe phenotype.^{33,34} We tested the hypothesis that A341V exacerbated the inhibitory effects of isoflurane on IKs. In addition, we identified a residue on KCNQ1 that plays a key role in the mechanism of inhalational anesthetic action.

Materials and Methods

Cell Culture and Transfection

The complementary DNA (cDNAs) of human KCNQ1 and KCNE1, the pore-forming and auxiliary subunits of IKs, respectively, were generous gifts from Dr. Michael Sanguinetti (University of Utah, Salt Lake City, UT). Three mutants of KCNQ1, A341V, F340A and F340C, were constructed using the QuikChange Site-Directed Mutagenesis Kit (Agilent, Palo Alto, CA) and confirmed by direct DNA sequencing. Fusion channel proteins of KCNQ1-KCNE1 were kindly provided by Dr. Robert Kass (Columbia University, New York, New York).³⁵ Briefly, the KCNQ1 and KCNE1 proteins were fused in different configurations with minimal peptide linkers between subunits to restrict protein interaction. The configurations were as follows: MK24 where the C-terminus of KCNE1 was linked to the N-terminus of KCNQ1, and MKK44 where the C-terminus of KCNE1 was linked to the N-terminus of a tandem homodimer of KCNQ1.

HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and Penicillin/Streptomycin in 5% CO₂ and 95% air at 37 °C. HL-1 cardiomyocytes were kindly provided by Dr. William Claycomb (Louisiana State University Health Science Center, New Orleans, LA) and cultured in Claycomb Medium (SAFC Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 10 µM noradrenaline and Penicillin/Streptomycin in 5% CO₂ and 95% air at 37 °C. Both cell lines were transiently transfected with the cDNAs using Lipofectamine 2000 CD (Invitrogen). Briefly, for a 35 mm dish of cells, 2.4 µg of human KCNQ1 cDNA and 2.4 µg of human KCNE1 cDNA were premixed with 0.8 µg of green fluorescent protein cDNA and Lipofectamine 2000 CD (ratio of cDNAs : Lipofectamine 2000 CD was 1 µg : 3 µl) in 0.4 ml serum-free medium and incubated 20 min at room temperature. The solution was then added to the dish and cells grown at 37 °C for 6 h, when medium was changed. After 2–3 days, the cells were trypsinized and used for recording. To record IKs current, only those cells showing green fluorescent protein fluorescence were used.

Solutions and Drugs

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Modified Tyrode solution contained 132 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl₂, 1.0 mM CaCl₂, 5 mM dextrose, and 10 mM HEPES (pH adjusted to 7.4 with NaOH). For experiments utilizing HEK293 cells, the external solution contained 132 mM N-methyl-D-glucamine, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 5 mM dextrose, and 0.1 mM KCl (pH adjusted to 7.4 with HCl). For experiments in HL-1 cardiomyocytes, the external

solution additionally contained 2 mM 4-aminopyridine (to block the transient outward potassium current), 0.2 mM CdCl₂ (to block the L-type calcium current), and 5 μ M E-4031 (to block IKr). The pipette solution contained 60 mM K-glutamate, 50 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 11 mM EGTA, 1 mM CaCl₂, and 5 mM K₂-adenosine triphosphate (pH adjusted to 7.4 with KOH).

Isoflurane (Baxter Healthcare, Deerfield, IL) was dispersed in the external solution, kept in glass-syringe reservoirs to ensure a constant concentration, and delivered to the recording chamber by using syringe pumps at a rate of 90 ml/h, followed by washing out with external solution at a rate of 90 ml/h. The volume of the recording chamber was approximately 0.6 ml, and complete solution changes in the chamber could be obtained within 4 minutes. At the end of each experiment, solution samples including isoflurane were collected and the concentrations in the recording chamber were measured using gas chromatography (GC8A chromatograph; Shimadzu, Kyoto, Japan), as previously reported.³⁶ The concentration of isoflurane used in this study was 0.54 ± 0.05 mM (mean $\pm$ SD), which is equivalent to 1.14 vol % at 22 °C. This is a clinically relevant concentration of isoflurane. We have previously shown that at this concentration, isoflurane increased action potential duration in ventricular myocytes isolated from guinea pig hearts.³⁶ This effect was attributed to the inhibition of IKs by isoflurane. At a higher supra-clinical concentration, isoflurane induced shortening of the action potential, likely due to its effect on the L-type calcium channel.

Electrophysiology

A drop of cells suspended in the modified Tyrode solution was placed in a flow-through chamber mounted on the stage of an inverted microscope. Patch pipettes were pulled from borosilicate glass capillaries (Garner Glass, Claremont, CA) with a micropipette puller (PC-10; Narishige, Tokyo, Japan) and heat-polished by using a microforge (MF-830; Narishige). The resistance of the recording pipettes, when filled with pipette solution and immersed in the modified Tyrode solution, ranged from 3 to 5 M Ω . Standard whole-cell configuration of the patch clamp technique was used to monitor currents. Gigaohm seal and rupture of the membrane were achieved in the modified Tyrode solution, followed by superfusion of the chamber with the external solution at a rate of 90 ml/h. Whole-cell currents were recorded at room temperature using the patch clamp technique with an Axopatch 200B amplifier and Digidata 1322A interface (Axon Instruments, Foster City, CA). pClamp10 software (Axon Instruments) was used for data acquisition and analysis. Current signal was sampled at 10 kHz and low-pass filtered at a cutoff frequency of 3 kHz. Depolarizing test pulses were applied from -60 mV to $+60$ mV in 20 mV increments. The duration of the depolarizing test pulses was 4 s and the holding potential was set at -50 mV.

Simulation of the action potential

Simulations of the cardiac action potential were run on MATLAB (The MathWorks, Natick, MA) using an established model for human ventricular myocytes that was developed by ten Tusscher et al.³⁷ Parameters for the IKs current were adjusted based on our experimental results. More complex models have been developed that take into consideration the cytosolic subspaces between the t-tubules and sarcoplasmic reticulum and more complex cytosolic calcium buffering,^{38,39} however the added complexity of these models does not alter or add

to the qualitative observations made with the ten Tusscher model. The model also exhibits adaptation of action potential duration (APD) showing a 10% reduction in APD at 90% repolarization (APD₉₀) between 1 and 3 Hz stimulation frequency. The parameters for IKs were modified according to the results obtained in this study. Specifically, to mimic the clinically relevant condition, changes in the whole-cell current in the HL-1 cardiac cells obtained under the heterozygous condition were incorporated into the model. Changes in current density was also included in the computational model since the heterozygous expression of the mutant A341V+KCNQ1+KCNE1 resulted in a significant smaller current density compared to the 2KCNQ1+KCNE1 wild-type.⁴⁰

Data Analysis and Statistical Analysis

The inhibition of current by isoflurane was quantified by the reduction of the peak current amplitude measured at the end of the depolarizing test pulses to +60 mV. To minimize the contribution of current rundown, only cells which showed recovery of IKs current after washout of isoflurane were used for analysis. The voltage dependence of IKs activation was determined by constructing an isochronal activation curve based on the peak current amplitude measured at the end of the depolarizing test pulses and normalized to the maximum current amplitude recorded during the test pulse to +60mV (I_{max}). The activation curves were fitted to a Boltzmann equation of the following form:

$I/I_{\max} = 1/[1 + \exp((V_{0.5} - V_t)/k)]$, where $V_{0.5}$ is the voltage of half-maximal activation, V_t is the test-pulse potential, and k is the slope factor. To determine the activating current kinetics, activation time to half-maximal amplitude ($T_{0.5}$) was measured during a depolarizing test pulse to +60 mV.

Data are presented as mean $\pm$ SE unless stated otherwise; n values indicate the number of experiments. Paired and unpaired two-tailed Student's t -tests and one-way ANOVA with the Scheffe's test for multiple pairwise comparisons were used for statistical analyses when appropriate. Randomization procedures were not used to assign the various groups. Power analysis was not conducted; the sample sizes were based on previous studies.^{40–42} Statistical analyses were performed using Origin 7 (OriginLab, Northampton, MA). In all comparisons, a value of $P < 0.05$ was considered statistically significant.

Results

Effects of Isoflurane on KCNQ1+KCNE1 and A341V+KCNE1 in HEK293 Cells

The co-assembly of KCNQ1 with its auxiliary subunit, KCNE1, recapitulates the native biophysical characteristics of IKs. This is demonstrated in Figure 1 (Panel A) that shows the representative wild-type IKs current traces in HEK293 cells which were co-transfected with wild-type KCNQ1 and KCNE1 (KCNQ1+KCNE1) recorded before and during application of isoflurane (0.54 ± 0.05 mM). The inhibitory effect of isoflurane on KCNQ1+KCNE1 was reversible. Isoflurane also induced a small but significant leftward shift in the isochronal activation curve accompanied by significant change in the slope factor (Figure 1B and Table 1). As shown in Figures 1C and 1D, HEK293 cells cotransfected with A341V and KCNE1 elicited a slowly-activating current with activation kinetics distinct from the wild-type, and also exhibited a unique steeply voltage-dependent characteristic (Table 1) as previously

reported.⁴⁰ The inhibitory effects of isoflurane on this mutant current were significantly greater than those on the wild-type, as summarized in Figure 1E. Isoflurane also had a greater impact on the isochronal activation curve of the mutant channel by inducing a more marked hyperpolarizing shift and increasing the slope factor (Figure 1D, Table 1). Thus, the overall isoflurane-induced changes were greater on the mutant A341V+KCNE1 than those observed on the wild-type KCNQ1+KCNE1.

Effects of Isoflurane on HEK293 cells under Heterozygous Conditions

Homozygous or heterozygous mutations are associated with inherited LQTS, although the latter are relatively more common. To mimic the heterozygous conditions, we expressed A341V with KCNQ1 and KCNE1 concurrently (2.4 μ g of cDNA for each subunit; A341V+KCNQ1+KCNE1), and compared it to the corresponding wild-type IKs which was transfected with 4.8 μ g of KCNQ1 and 2.4 μ g of KCNE1 cDNA (2KCNQ1+KCNE1). Figure 2A and 2B show the representative current traces and corresponding isochronal activation curves of the wild-type 2KCNQ1+KCNE1 in control and in the presence of isoflurane. Isoflurane inhibited 2KCNQ1+KCNE1 and had a small, but statistically significant effect on the isochronal activation curve where $V_{0.5}$ increased from 21.8 to 23.5 mV (Figure 2A and B; Table 1). The heterozygous combination of A341V+KCNQ1+KCNE1 produced slowly activating voltage-dependent currents with kinetics distinct from the wild-type 2KCNQ1+KCNE1, and exhibited greater inhibition by isoflurane (Figures 2C; Table 1). Isoflurane also induced a significant leftward shift in the isochronal activation curve and a small but significant increase in the slope factor in the A341V+KCNQ1+KCNE1 (Figure 2D; Table 1). These results revealed that the A341V mutant construct is more sensitive to isoflurane than the wild-type IKs even under heterozygous conditions that mimic the clinical setting, as summarized in Figure 3A. In addition, the current density was lower for the A341V+KCNQ1+KCNE1 than for 2KCNQ1+KCNE1 or KCNQ1+KCNE1 (Figure 3B). The latter comparison suggested that A341V exerted a dominant negative effect on the resultant current.

Effects of Isoflurane on KCNQ1+KCNE1 and A341V+KCNE1 in HL-1 cardiomyocytes

Though the utilization of various cell lines is commonly used in the study of ion channel function, the biophysical characteristics of IKs mutants may be dependent on the expression system used.^{34,43,44} Furthermore, our results obtained in a mammalian non-cardiac cell line could potentially be altered when investigated in a cardiac environment. Consequently, we tested whether the effects of isoflurane on the wild-type and mutant IKs were expression system-dependent. Mutant and wild-type IKs channel complexes were expressed in the HL-1 cell line, which is derived from the AT-1 mouse atrial cardiomyocyte lineage,^{45,46} and the results were compared to those obtained from the HEK293 cells. Figure 4A shows the representative current traces of KCNQ1+KCNE1 expressed in HL-1 cardiomyocytes. As expected, KCNQ1+KCNE1 resulted in IKs with the characteristic slow activation kinetics, and this current was inhibited by isoflurane. However, unlike in HEK293 cells, isoflurane did not change $V_{0.5}$ and k significantly in HL-1 cardiomyocytes expressing KCNQ1+KCNE1 (Figure 4B; Table 2). In contrast, A341V+KCNE1 in HL-1 cells exhibited biophysical characteristics distinct from the wild-type and similar to the results obtained in HEK293 cells (Figure 4C and D; Table 2). The isoflurane-induced shift in the isochronal

activation curve and change in the slope factor in the mutant channel expressed in the HL-1 cells were similar to those in the HEK293 cells. Isoflurane inhibited the wild-type KCNQ1+KCNE1 expressed in HL-1 cells by $19.5 \pm 2.7 \%$, as summarized in Figure 4E. Interestingly, this effect was significantly smaller than those we observed when KCNQ1+KCNE1 were co-expressed in HEK293 cells. The mutant A341V+KCNE1 was significantly more sensitive to inhibition by isoflurane compared to the wild-type IKs when expressed in HL-1 cells, similar to the results obtained in HEK293 cells (Figure 4E). Thus, in the two cell lines used, the effects of isoflurane on A341V+KCNE1 were similar.

Effects of Isoflurane on HL-1 cardiomyocytes under Heterozygous Conditions

Experiments were also repeated to investigate the effects of isoflurane under heterozygous conditions in HL-1 cells. Under these conditions, our results showed that the effects of isoflurane on the wild-type and mutant IKs channel constructs were diminished compared to those in the HEK293 cells. Figure 5 shows the inhibitory effects of isoflurane on the wild-type IKs (2KCNQ1+KCNE1) and mutant A341V+KCNQ1+KCNE1 in HL-1 cardiomyocytes. For the wild-type, isoflurane inhibited 2KCNQ1+KCNE1 by $16.3 \pm 3.1 \%$ (Figure 5E), which was significantly less than the inhibitory effect observed using HEK293 cells. In addition, isoflurane had a small but significant effect on the slope factor of the isochronal activation curve of the wild-type IKs expressed in HL-1 cells, in contrast to the effect in HEK293 cells (Table 2). Isoflurane inhibited the mutant A341V+KCNQ1+KCNE1 by $30.5 \pm 4.0 \%$ (Figure 5E), which was also significantly less than that observed in HEK293 cells. Furthermore, in contrast to the mutant expressed in HEK293 cells, isoflurane did not significantly impact $V_{0.5}$ and k when the mutant channel was expressed in HL-1 cells (Table 2). Nevertheless, the key observation that the mutant channel in a heterozygous environment was more sensitive to inhibition by isoflurane than the wild-type was consistently observed in both cell types.

Site of isoflurane action

Our results show that the inhibitory effect of isoflurane was greater on IKs with the A341V mutation compared to the wild type. The underlying molecular mechanism is unknown. In fact, the fundamental mechanism of inhalational anesthetic action on IKs is not known. Based on our findings, we hypothesized that the anesthetic interaction site was on KCNQ1. Thus, we investigated the inhibitory effect of isoflurane on HL-1 cells transiently transfected with KCNQ1 in the absence of KCNE1. Figure 6A shows that in the presence of isoflurane (0.5 mM), a marked inhibition of the wild-type KCNQ1 current was observed. In a representative recording, the KCNQ1 current was inhibited by approximately 90% by the anesthetic. The effect was reversible upon washout of isoflurane (data not shown). No shift in the normalized isochronal activation curves was observed in the presence of isoflurane (Figure 6B). The $V_{1/2}$ for half-maximal activation and slope factor was -1.8 ± 2.4 mV and 21.9 ± 2.4 in control and 1.4 ± 3.4 mV and 21.5 ± 3.3 in the presence of isoflurane, respectively ($n=9$). Summary of the inhibitory effects of isoflurane on KCNQ1 at various test potentials are depicted in Figure 6C. This effect on KCNQ1 was significantly greater than that on KCNQ1+KCNE1. These results supported our hypothesis that the isoflurane interaction site is on the KCNQ1 α -subunit.

We also hypothesized that an isoflurane interaction site is in the vicinity of the A341 residue. A candidate site was F340 on KCNQ1, a residue that is critical to the functional interaction between KCNQ1 and KCNE1,^{47,48} and also to the channel's interaction with IKs antagonists.^{49,50} Substitution of F340 with alanine or cysteine attenuated the inhibitory effects of isoflurane. As shown in Figure 7, the F340A and F340C mutants of KCNQ1 were significantly less sensitive to isoflurane compared to the WT KCNQ1 (Figure 6). Differences were also observed between F340A and F340C, with F340A being less sensitive to isoflurane. Since A341V expressed in HL-1 cells in the absence of KCNE1 did not form a functional channel, the direct effects of isoflurane on this mutant could not be assessed.

Impact of KCNE1 on anesthetic action

Our result that the effect of isoflurane on KCNQ1 was greater in the absence of the KCNE1 subunit suggested that KCNE1 modulated the anesthetic effect. In order to investigate whether KCNE1 could potentially modulate the accessibility of isoflurane to its interaction site on KCNQ1, we utilized fusion proteins of KCNQ1 and KCNE1, whereby the subunits were fused with minimal peptide linkers.³⁵ These are schematically presented in Figure 8A. In the MK24 protein, the linkage between the KCNQ1 and KCNE1 subunits forced a 1:1 stoichiometry. In the MKK44 protein, an additional KCNQ1 was fused to MK24 that resulted in a final KCNQ1:KCNE1 ratio of 2:1 (2KCNQ1+KCNE1). Sample traces and the effect of isoflurane from HL-1 cells transiently transfected with either MK24 or MKK44 are demonstrated in Figure 8B, and the results are summarized in Figure 8C. The inhibitory effect of isoflurane was significantly greater on MKK44 than on MK24. This difference is likely due to an additional available anesthetic binding site on the “extra” KCNQ1 in the MKK44 fusion protein.

Simulation of the Impact of Isoflurane and A341V on the Ventricular Action Potential

To determine the impact of isoflurane and of A341V on the ventricular action potential, simulations were conducted based on the model developed by ten Tusscher. The heterozygous conditions (Figure 5) were incorporated into the model. These simulations are shown in Figure 9 for the wild-type and mutant in the absence and presence of isoflurane. As predicted based on the biophysical parameters, the A341V mutation resulted in an APD₉₀ of 355 ms compared to the ADP₉₀ of 285 ms with the wild-type IKs. However, the effects of isoflurane on the APD were relatively modest for both cases, where APD₉₀ increased to 294 ms with the wild-type IKs and to 365 ms with the A341V.

Simulations were then run using different stimulation frequencies. For the wild-type IKs, the integrity of the action potential profile was relatively unchanged in the presence or absence of isoflurane at stimulation frequencies of 1, 2, and 3 Hz (Figure 10A). Thus, the relatively modest increase in APD₉₀ by isoflurane did not trigger early afterdepolarizations (EADs) up to a stimulation frequency of 3 Hz. Stimulation frequencies at 4 Hz and greater did trigger EADs (data not shown). Whether isoflurane facilitated A341V-induced EADs was also tested by running simulations at stimulation frequencies of 1–3 Hz (Figure 10B). Our computational modeling revealed that at 3 Hz, A341V triggered EADs, similar to the result we had previously reported.⁴⁰ The EADs were also persistent in the presence of isoflurane.

However, at the lower stimulation frequencies of 1 and 2 Hz, isoflurane did not facilitate the triggering of EADs in an action potential model incorporated with the properties of A341V.

Discussion

In this study, we report that the inhibitory effect of isoflurane on a KCNQ1 mutation, A341V, associated with an inherited form of LQT1, is greater than on the corresponding wild-type channel. This key finding was consistent under homozygous and heterozygous conditions, and in two different cell lines, one of which was derived from a cardiac lineage. We believe that our study is the first to report that the effects of isoflurane are altered in a LQT1 associated mutant of KCNQ1, the pore-forming region of the IKs channel.

The A341V mutation is one of the most common mutations found in congenital LQT1.³³ However, the correlation of its severe phenotype to the A341V-induced electrophysiological changes has been confounding. In our study, when HL-1 or HEK293 cells were utilized for transfection, the current densities were significantly lower in A341V+KCNE1 and A341V+KCNQ1+KCNE1 compared to the respective wild-type combinations. This is suggestive of a dominant negative effect of A341V.⁴⁰ Similarly, Brink et al. reported that A341V exerted a dominant negative effect in the CHO cell line.³⁴ However, Kobori et al. reported that A341V was a loss-of-function type mutation and exerted no dominant negative effect in COS7 cells.⁴³ Wang et al. also reported that A341V exhibited little dominant negative effect in *Xenopus* oocytes.²⁷ These studies suggested that the A341V subunit was likely unable to co-assemble with wild-type KCNQ1 subunits under heterozygous conditions. Additionally, we have recently reported on a novel finding that in the cardiac HL-1 cell line, transfection of A341V did not result in a functional channel, but the KCNE1 β -subunit partially restored function when co-expressed together with altered biophysical properties.⁴⁰ This was also evident in a non-cardiac cell line, HEK293, in this study.

Previously, we reported that isoflurane induced a negative shift of the voltage dependence of IKs activation in guinea pig ventricular myocytes.²² In the present study, isoflurane shifted the voltage dependence of wild-type IKs (KCNQ1+KCNE1) and also of A341V+KCNE1 in the negative direction when expressed HEK293 cells. Such a shift would suggest an increase in current by isoflurane at depolarizing potentials, as opposed to the observed inhibitory effects by the volatile anesthetic. Since the overall isoflurane effect on IKs was inhibitory, the observed shift in activation could potentially attenuate the inhibition, but evidently was not the predominant effect. Interestingly, on the other hand, isoflurane shifted the activation curve of 2KCNQ1+KCNE1 in the positive direction in both HEK293 and HL-1 cells. Though the reported shifts were statistically significant, these changes were small relative to the larger impact the A341V mutation had on the inhibition of current amplitude and activation kinetics (as measured by $T_{0.5}$, the time to half-maximal peak) by isoflurane. Thus, the contribution of the shifts in activation will likely be masked by the larger effect of isoflurane on IKs current amplitude.

Although the molecular mechanism underlying the enhanced effect of isoflurane on A341V is unknown, the region surrounding this residue may be important for the pharmacological properties of IKs. Studies have shown that several residues located in KCNQ1 located in this

S6 transmembrane region of the channel affect the pharmacology of the IKs channel. For example, an A344V mutation in KCNQ1 increased the sensitivity of IKs to the inhibitory effects of local anesthetics.⁵¹ Mutation of residues at position 337 and 340 dramatically altered the sensitivity of KCNQ1 to the inhibitory effects of chromanol 293B, an IKs blocking agent with antiarrhythmic properties.⁴⁹ Residues at position 337, 339, 340 and 344 were critical to the binding of a benzodiazepine derivative, L-735821 (L-7), a potent blocker of KCNQ1.⁵⁰ Additionally, a KCNQ1 mutant, A341C, was found to be significantly more resistant to the inhibitory effects of L-7 than wild-type KCNQ1.⁵⁰ Since A341V resulted in a nonfunctional channel, the impact of this mutation on the L-7 effects cannot be speculated.⁴⁰ Nevertheless, alanine at position 341 and the neighboring residues in the S6 region of KCNQ1 appear to be an important determinant of the pharmacological properties of IKs.

The complexity of delineating the mechanism underlying the enhanced sensitivity of the A341V mutant of IKs is compounded, because the fundamental molecular mechanism of inhalational anesthetic action on IKs is not known. To our knowledge, this is the first study to reveal that the F340 residue is a critical determinant of isoflurane action. Since the effect of the anesthetic was not completely abrogated in both the F340A and F340C mutants, other residues are likely involved. Our results did not discriminate whether this residue directly interacted with isoflurane, or whether it served as a critical component in shaping the anesthetic binding region. Interestingly, substitution of F340 with an alanine was more effective in attenuating the isoflurane effect than with the cysteine. Since both phenylalanine and alanine are non-polar, whether the uncharged polar nature of cysteine contributed to the differential effect remains to be determined.

We hypothesized that KCNE1 modulated the anesthetic action. This is, in part, supported by the fusion protein studies. The inhibitory effect of isoflurane was greater on the MKK44 protein with a KCNQ1:KCNE1 stoichiometry of 2:1 than on MK24 with a stoichiometry of 1:1, which is likely due to the additional isoflurane interaction site on the “extra” KCNQ1 in the MKK44 protein. An intriguing observation was that the inhibitory effect of isoflurane on HL-1 cells transfected with MKK44 was closer to that of HL-1 cells co-transfected with KCNQ1 and KCNE1 than to that of MK24. The native stoichiometry of KCNQ1 to KCNE1 has been intensely debated, and ranges from 1:1 to 4:2, with possibilities of a varying stoichiometry.^{35,52–56} Our results would appear to support a 4:2 stoichiometry. However, this is highly speculative since one would need to consider potential spatial restriction imposed on the interaction between KCNQ1 and KCNE1 due to the linkers in the fusion proteins.

The modulation of volatile anesthetic action by an ion channel’s auxiliary β -subunit is a novel finding. This contradicts a previous finding that showed the inability of KCNE1 to modulate anesthetic effect on IKs when expressed in *Xenopus* oocytes.⁵⁷ The discrepancy is likely due to the difference in the expression system used, since a cardiac cell line was utilized to record current in a cardiac environment in the present study. The role of KCNE1 in modulating pharmacological actions on IKs has also been demonstrated for the inhibitors, L-7 and chromanol 293B.^{50,49,58}

The impact of our molecular and cellular findings on the action potential was investigated by simulations of the action potential. Computational modeling of the action potential confirmed the predicted prolongation of the APD based on the effects of isoflurane on IKs. The isoflurane-induced prolongation was relatively modest for both the wild-type and mutant groups. The model also revealed that for the wild-type IKs, the isoflurane-induced prolongation did not result in an arrhythmogenic profile of the action potential with stimulation frequencies up to 3 Hz. For the A341V mutant, the high frequency of 3 Hz triggered persistent EADs, which were slightly more pronounced in the presence of isoflurane. Thus, isoflurane will be predicted to exacerbate the EADs triggered by the mutation. Our modeling was based on the effects of isoflurane on IKs alone, and did not factor in the impact of the anesthetic on other potassium channels, particularly IKr, that could significantly impact the repolarization reserve. Thus the simulations likely err on the side of underestimating the impact of isoflurane on the A341V mutant. Additionally, our results are dependent on the initial IKs current density set in the ten Tusscher model.³⁷ Thus, if the reliance on IKs for ventricular repolarization is greater in the clinical setting, then our model will be an underestimate of the effects of isoflurane on the APD.

IKs is also highly regulated by β -adrenergic stimulation.^{59,60} Consequently, another limitation of our AP simulation is that changes in sympathetic input were not incorporated. Sympathetic stimulation could influence the sensitivity of IKs to isoflurane, but this is not well characterized. We have previously reported on a preliminary finding that isoflurane can sensitize and de-sensitize the IKs to sympathetic input, which was dependent on the stimulation pathway.⁶¹ Since isoflurane was found to sensitize IKs to forskolin, and desensitize the channel to isoproterenol, the observation was attributed to multiple effects of the anesthetic along the signaling pathway. The complexity of this issue is further magnified by a recent study that reported on defects in phosphorylation that accompanied the A341V mutation in KCNQ1.²⁹ Thus, the overall effect of isoflurane on IKs and LQT1-associated mutants, and ultimately on the action potential, would need to be determined in the setting of changes in sympathetic input.

There are other limitations in this study. We investigated only a single mutation of A341V although compound heterozygous mutations are relatively common in inherited LQTS.^{32,62} In addition, other volatile anesthetics such as sevoflurane and desflurane may exhibit differential effects. Furthermore, there still remains a considerable gap in translating bench top results to the clinical setting. The use of patient-specific, induced pluripotent stem cell-derived cardiomyocytes in studying inherited LQTS should facilitate a more definitive delineation of the effects of isoflurane on IKs.⁶³

Acknowledgments

We thank Ann Tobin, Ph.D. while at the Medical College of Wisconsin (Milwaukee, WI, USA; Department of Anesthesiology) for assisting in the initial set of site-directed mutagenesis for the F340 mutant constructs.

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Figure 1.

The impact of isoflurane on wild-type IKs (KCNQ1+KCNE1) and A341V mutant in HEK293 cells. (A) Representative whole-cell current traces of KCNQ1+KCNE1 in the absence and presence of isoflurane (0.54 ± 0.05 mM; equivalent to 1.14 vol % at 22 °C) are shown. The voltage protocol is shown in the inset. (B) The effects of isoflurane on the voltage dependence of activation of KCNQ1+KCNE1. The isochronal activation curves were fitted with the Boltzmann equation as described in Materials and Methods under Data Analysis and Statistical Analysis ($n = 9$; Table 1). (C) Representative whole cell current

traces of A341V+KCNE1 in control and in the presence of isoflurane are shown. The voltage protocol was as shown in Figure 1A. A341V+KCNE1 exhibited markedly slow activation kinetics compared to the wild-type IKs. The inhibitory effects of isoflurane on A341V+KCNE1 were greater than those on KCNQ1+KCNE1. (D) The effects of isoflurane on the voltage dependence of activation of A341V+KCNE1 (n = 8; Table 1). (E) Summary of the inhibitory effects of isoflurane on KCNQ1+KCNE1 and A341V+KCNE1 in HEK293cells. The inhibition of current by isoflurane was quantified by the reduction of the peak current amplitude measured at the end of the depolarizing test pulses to +60 mV. The mutation of alanine to valine at position of 341 of KCNQ1 significantly enhanced the inhibitory effects of isoflurane on IKs (n = 8–9, * $P < 0.01$ versus KCNQ1+KCNE1).

Figure 2.

The impact of isoflurane on wild-type IKs and A341V mutant under heterozygous conditions. Whole-cell currents were monitored in HEK293 cells expressing A341V +KCNQ1+KCNE1 (2.4 μ g of complementary DNA [cDNA] for each subunit) and compared to those with 2KCNQ1+KCNE1 (4.8 μ g of KCNQ1 and 2.4 μ g of KCNE1 cDNA). (A) Representative current traces of 2KCNQ1+KCNE1 in control and in the presence of isoflurane are shown. (B) The effects of isoflurane on the voltage dependence of activation of 2KCNQ1+KCNE1. The isochronal activation curves were fitted with the Boltzmann equation as described in Materials and Methods under Data Analysis and Statistical Analysis ($n = 11$; Table 1). (C) Representative current traces of A341V+KCNQ1+KCNE1 in control and in the presence of isoflurane are shown. The inhibitory effects of isoflurane on A341V +KCNQ1+KCNE1 were greater than those on 2KCNQ1+KCNE1, similar to the comparisons between KCNQ1+KCNE1 and A341V+KCNE1. (D) The effects of isoflurane on the voltage-dependence of activation of A341V+KCNQ1+KCNE1 is depicted ($n = 13$; Table 1).

Figure 3.

Summary of the inhibitory effects of isoflurane under heterozygous conditions and the impact of A341V on current density. (A) Summary of the inhibitory effects of isoflurane on 2KCNQ1+KCNE1 and A341V+KCNQ1+KCNE1 at +60 mV. Similar to the homozygous conditions (Figure 2), the A341V mutant was significantly more susceptible to the inhibitory effects of isoflurane ($n = 11-13$, $*P < 0.01$ versus 2KCNQ1+KCNE1). (B) The current-voltage relationships under control (isoflurane-free) conditions. Current amplitudes were normalized to cell capacitance to yield current density. A341V+KCNQ1+KCNE1 exhibited significantly smaller current density than the corresponding wild-type IKs, 2KCNQ1+KCNE1, and KCNQ1+KCNE1 ($n = 11-13$, $*P < 0.01$ vs. 2KCNQ1+KCNE1, $P <$

0.05 vs. KCNQ1+KCNE1; §P < 0.05 vs. 2KCNQ1+KCNE1 and KCNQ1+KCNE1). This is suggestive of a dominant negative effect by A341V.

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Figure 4.

The impact of isoflurane on KCNQ1+KCNE1 and A341V+KCNE1 in a cardiac cell line, HL-1 cells. (A) Representative whole-cell current traces of KCNQ1+KCNE1 in control and in the presence of isoflurane is shown. The voltage protocol is as depicted in Figure 1A. (B) The effects of isoflurane on the corresponding voltage-dependence of activation of KCNQ1+KCNE1 are depicted. The isochronal activation curves were fitted with the Boltzmann equation as described in Materials and Methods under Data Analysis and Statistical Analysis ($n = 8$; Table 2). In contrast to the results in HEK293 cells, isoflurane did not shift the activation curve of KCNQ1+KCNE1 in HL-1 cells. (C) Representative whole-cell current traces of A341V+KCNE1 in control and in the presence of isoflurane is shown. (D) The effects of isoflurane on the voltage-dependence of activation of A341V+KCNE1 are presented ($n = 8$; Table 2). (E) Summary of the inhibitory effects of isoflurane

on KCNQ1+KCNE1 and A341V+KCNE1 in HL-1 cells. Similar to the results in HEK 293 cells, A341V+KCNE1 was significantly more sensitive to the inhibitory effects of isoflurane compared to those on KCNQ1+KCNE1 (n = 8/group, $*P < 0.01$ versus KCNQ1+KCNE1).

Figure 5.

The effects of isoflurane on wild-type IKs and A341V mutant under heterozygous conditions in HL-1 cells. The cDNA ratios for each subunit used for transfection were the same for those used in Figure 2. (A) Representative current traces of 2KCNQ1+KCNE1 recorded in control and in the presence of isoflurane are shown. (B) The corresponding effects on the voltage-dependence of activation of 2KCNQ1+KCNE1. The isochronal activation curves were fitted with the Boltzmann equation as described in Materials and Methods under Data Analysis and Statistical Analysis ($n = 10$; Table 2). Isoflurane shifted the activation curve in the depolarizing direction. (C) Representative current traces of A341V+KCNQ1+KCNE1 in control and in the presence of isoflurane are shown. (D) The corresponding effects on the voltage dependence of activation of A341V+KCNQ1+KCNE1 ($n = 8$). The isochronal activation curves were not significantly shifted by the application of isoflurane, which was in contrast to the results in HEK293 cells (see Figure 3). (E) Summary of the inhibitory effects of isoflurane on 2KCNQ1+KCNE1 and A341V+KCNQ1+KCNE1 in HL-1 cells. Similar to the results in HEK293 cells, the A341V mutant was significantly more sensitive to the inhibitory effects of isoflurane compared to corresponding wild-type IKs ($n = 8-10$, $*P < 0.01$ versus 2KCNQ1+KCNE1).

Figure 6.

The effect of isoflurane on KCNQ1 expressed alone in HL-1 cells. (A) Representative current traces of KCNQ1 are shown in the absence and presence of 0.5 mM isoflurane. (B) The effects of isoflurane on the corresponding voltage-dependence of activation of KCNQ1 are depicted. Isoflurane had no significant effect on the KCNQ1 isochronal activation curves that were fitted with the Boltzmann equation as described in Materials and Methods under Data Analysis and Statistical Analysis. (C) Summary of the inhibitory effects of isoflurane on KCNQ1 at various test potentials is shown.

Figure 7.

Role of F340 in determining anesthetic sensitivity. The inhibitory effects of isoflurane (0.5 mM) were determined in two KCNQ1 mutants, F340A and F340C, as shown in panels (A) and (B), respectively. The results are summarized in (C). Current inhibition was determined at a test-pulse potential of +60 mV from a -50 mV holding potential. Compared to the wild type KCNQ1, the inhibition by isoflurane on the two mutants were significantly attenuated. However, between the two mutants, a significant difference was observed in the inhibition by isoflurane. *denotes significantly different from F340A, n=4-5/group.

Figure 8.

Effects of isoflurane on whole-cell potassium currents (IKs) fusion proteins. **A.** Schematic of the fusion proteins. In the MK24 protein, the C-terminus of KCNE1 was linked to the N-terminus of KCNQ1, and in the MKK44, the C-terminus of KCNE1 was linked to the N-terminus of a tandem homodimer of KCNQ1. **B.** Isoflurane effects on MK24 and MKK44. In control, both MK24 and MKK44 exhibited IKs-like activation kinetics. **C.** Summary of the effects of isoflurane (0.5 mM) on the fusion proteins. Inhibition of current was determined at a test-pulse potential of +60 mV from a -50 mV holding potential. *denotes significantly different from MK24, n=9-12/group.

Figure 9. Simulation of the cardiac action potential using the ten Tusscher model of human ventricular myocyte. (A) Simulated action potential under control condition (wild-type IKs) in the absence and presence (dashed lines) of isoflurane. (B) Simulated action potential under LQT1 (Long QT Syndrome Type 1) conditions with heterozygous expression of A341V in the absence and presence (dashed lines) of isoflurane. In both cases, isoflurane triggered prolongation of the action potential duration.

Figure 10.

Simulation of the cardiac action potential at various stimulation frequencies under control and LQT1 conditions with heterozygous expression of A341V. Using the ten Tusscher model, ventricular action potentials under control (wild-type IKs; Panel A) and LQT1 (A341V; Panel B) conditions were stimulated at 1, 2, and 3 Hz in the absence and presence of isoflurane. For the wild-type conditions, the integrity of the action potential profile was maintained at the various stimulation frequencies; no evidence of arrhythmogenicity was evident in the absence or presence of isoflurane. For the LQT1 conditions, no evidence of arrhythmogenicity was evident at stimulation frequencies of 1 and 2 Hz. However, at 3 Hz, early afterdepolarizations were apparent in the absence and presence of isoflurane.

Table 1

Biophysical Variables of A341V Constructs and Corresponding Wild-type IKs in HEK-293 cells

	V _{0.5}		k		T _{0.5}	n
	control	isoflurane	control	isoflurane		
KCNQ1+KCNE1	26.3 ± 1.8	23.5 ± 1.5 *	13.3 ± 0.7	14.6 ± 0.6 *	0.78 ± 0.09	9
A341V+KCNE1	41.5 ± 0.6	34.1 ± 1.5 *	5.4 ± 0.6	10.4 ± 0.6 *	1.25 ± 0.11 †	8
2KCNQ1+KCNE1	21.8 ± 1.4	23.5 ± 1.5 §	14.6 ± 0.5	14.6 ± 0.5	0.29 ± 0.03	11
A341V+KCNQ1+KCNE1	30.4 ± 1.1	27.5 ± 1.0 *	11.8 ± 0.4	12.7 ± 0.4 *	0.72 ± 0.06 ‡	13

V_{0.5} (voltage of half-maximal activation), k (slope factor), and T_{0.5} (activation time to half-maximal amplitude) were determined as described in Materials and Methods under Data Analysis and Statistical Analysis. Statistical comparisons of V_{0.5} and k were performed between control and isoflurane within the same group. Data are presented as mean ± SE.

* $P < 0.01$ vs. control,

§ $P < 0.05$ vs. control,

† $P < 0.01$ vs. KCNQ1+KCNE1,

‡ $P < 0.01$ vs. 2KCNQ1+KCNE1

Biophysical Variables of A341V Constructs and Corresponding Wild-type IKs in HL-1 cardiomyocytes

Table 2

	$V_{0.5}$		k		n
	control	isoflurane	control	isoflurane	
KCNQ1+KCNIE1	29.4 ± 1.7	30.2 ± 1.6	12.3 ± 0.5	12.2 ± 0.7	8
A341V+KCNIE1	41.5 ± 1.0	38.8 ± 1.6 §	5.4 ± 1.0	7.7 ± 1.0 §	8
2KCNQ1+KCNIE1	22.8 ± 1.7	25.6 ± 1.8 §	14.6 ± 0.6	13.6 ± 0.6 §	10
A341V+KCNQ1+KCNIE1	31.1 ± 1.4	31.3 ± 1.1	11.7 ± 0.5	11.5 ± 0.4	8

$V_{0.5}$ (voltage of half-maximal activation) and k (slope factor) were determined as described in Materials and Methods under Data Analysis and Statistical Analysis. Statistical comparisons were performed between control and isoflurane within the same group. Data are presented as mean ± SE.

§ $P < 0.05$ vs. control