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Type 2 ryanodine receptors are highly sensitive to alcohol

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Abstract

Exposure to ethanol levels reached in circulation during alcohol intoxication (≥10 mM) constricts cerebral arteries in rats and humans. Remarkably, targets and mechanisms underlying this action remain largely unidentified. Artery diameter is regulated by myocyte Ca^{2+} sparks, a vasodilatory signal contributed to by type 2 ryanodine receptors (RyR2). Using laser confocal microscopy in rat cerebral arteries and bilayer electrophysiology we unveil that ethanol inhibits both Ca^{2+} spark and RyR2 activity with IC_{50}<20 mM, placing RyR2 among the ion channels that are most sensitive to ethanol. Alcohol directly targets RyR2 and its lipid microenvironment, leading to stabilization of RyR2 closed states.

Keywords

ryanodine receptor; Ca^{2+} spark; alcohol; cerebral artery

Introduction

Moderate to heavy episodic alcohol intake is associated with an increased risk for cerebral artery constriction and disease [1,2,3,4]. Alcohol-induced cerebrovascular disease and modification of vascular physiology is independent of beverage type but linked to ethanol (EtOH) itself [1,5,6,7]. Acute exposure to EtOH levels reached in circulation during alcohol intoxication (10–100 mM) constricts cerebral arteries in several species, including rats and humans [7,8,9,10,11]. However, targets and mechanisms underlying EtOH constriction of cerebral arteries remain largely unidentified.

Cerebral artery diameter and myogenic tone are heavily dependent on the activity of large conductance, Ca^{2+}- and voltage-gated K^+ (BK) channels. BK channels generate spontaneous transient outward currents (STOCs), which drive the myocyte membrane potential towards

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more negative values and reduce voltage-dependent Ca^{2+}-entry, opposing vasoconstriction and promoting dilation [12,13]. Using rat and mouse models, we showed that BK current inhibition by EtOH is linked to alcohol constriction of cerebral arteries [9,10,14]. In cerebral artery smooth muscle, however, BK channel-generated STOCs are activated by subsarcolemmal Ca^{2+} sparks, vasodilating Ca^{2+} signals that result from sarcoplasmic reticulum (SR) ryanodine receptor (RyR) activation [13,15]. We found that EtOH constriction of cerebral arteries is greatly reduced in absence of the BK beta1 subunit [10], an accessory channel protein that functionally couples BK channels to Ca^{2+} sparks in cerebral artery smooth muscle [16]. We also documented that acute exposure of rat cerebral arteries to 50 mM EtOH, a concentration well above blood alcohol levels (BAL) that constitute legal intoxication (0.08 g/dl or ~18 mM; www.niaaa.org), reduced Ca^{2+} spark frequency in cerebral artery myocytes without altering other Ca^{2+} signaling modalities, such as IP_3-stimulated Ca^{2+} waves [9]. Collectively, these previous findings led to the hypothesis that cerebral artery smooth muscle RyRs are a target of alcohol, with EtOH levels found in circulation during alcohol intoxication leading to RyR inhibition and Ca^{2+} spark suppression.

RyRs result from tetrameric association of homomeric subunits, three isoforms being identified: RyR1, RyR2 and RyR3 [17,18,19]. The functional RyR type that predominates in the subsarcolemmal SR of rat resistance-size cerebral artery myocytes is RyR2 [20]. Here we use lipid bilayer electrophysiology and Ca^{2+} confocal imaging to demonstrate for the first time the sensitivity of recombinant RyR2 and Ca^{2+} sparks in cerebral arteries to intoxicating and clinically relevant EtOH levels.

Material and Methods

Artery isolation and Ca^{2+} spark recordings

Male adult Sprague–Dawley rats (~250 g) were decapitated with a guillotine following institutionally-approved procedures. Brains were removed and placed in ice-cold (4 °C) HEPES-buffered physiological saline solution (PSS) containing (mM): 134 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (pH 7.4). Ca^{2+} spark recordings from posterior cerebral and cerebellar arteries, and data analysis were conducted as outlined in Supplementary Information (SI) following previous methods [21,22].

Cell culture and SR preparation

RyR2-pcDNA3 was kindly provided by Dr. Wayne Chen (U. Calgary). HEK293 cells were transiently transfected with RyR2 cDNA inserted into pcDNA3 following previous procedures [23]. Transfected cells were processed as described elsewhere [24] to render a crude SR suspension, which was aliquoted, frozen in liquid nitrogen, and stored at −80ºC.

Electrophysiology

RyR2 were reconstituted in planar bilayers cast of POPE:POPS:POPC, 5:3:2 (wt/wt/wt) following procedures identical to those used to reconstitute cerebral artery SR native RyR [20]. Electrophysiological recordings and data analysis were performed as described in SI.

Chemicals

Bilayer lipids were purchased from Avanti; other chemicals were purchased from Sigma-Aldrich. Stock solutions and dilution to final concentration are described in SI.

Results

Ethanol levels found in circulation during alcohol intoxication suppress Ca^{2+} sparks

Extending an early finding [9], a 3 min exposure of intact cerebral arteries to EtOH levels matching BAL reached during moderate-heavy alcohol intake (50 mM) had no effect on Ca^{2+} spark amplitude but drastically reduced Ca^{2+} spark frequency. Ethanol action was fully reversible upon a 5 min washout of the artery with EtOH-free PSS (Fig. 1A–C). Moreover, EtOH (10–50 mM) decreased Ca^{2+} spark frequency in a concentration-dependent manner, with EtOH concentrations matching BAL that constitutes legal intoxication in most US states (~18 mM) decreasing Ca^{2+} spark frequency by 30% ($p<0.007$). From fitting the data to a three-parameter sigmoidal equation, IC_{50} for EtOH inhibition of Ca^{2+} spark frequency was calculated at 17±1.1 mM (Fig. 1D). These data demonstrate that in intact rat cerebral arteries, Ca^{2+} sparks, a vasodilatory signal generated by smooth muscle RyR activity, is reversibly inhibited by EtOH levels that have been: i) reported to reversibly constrict cerebral arteries in our experimental model [9], ii) linked to cerebrovascular constriction and disease in humans [1,2,3,4,7,8], and iii) found in human circulation after moderate-heavy alcohol intoxication [25](www.niaaa.org).

Data from intact cerebral arteries raise two related questions: first, whether EtOH-induced inhibition of Ca^{2+} sparks is mediated by EtOH itself or cell-generated EtOH metabolites/byproducts of alcohol presence, such as acetaldehyde or reactive oxygen species (ROS), which are known to modulate RyR activity [26,27]. Second, whether EtOH action on Ca^{2+} sparks results from a direct interaction between drug and the RyR receptor protein complex or, rather, from drug-induced targeting of cytosolic signaling which, in turn, alters RyR function. Considering that: 1) ion channel reconstitution into planar phospholipid bilayers is widely used to study the biophysics and pharmacology of both native and recombinant RyR [28,29,30], 2) we demonstrated that RyR2 protein prevailed in the sub-plasmalemma of rat cerebral artery myocytes, a region enriched in SR and where the BK-RyR functional complex clusters in cerebral arteries [13,20,31], and 3) we showed that following ion channel reconstitution into POPE:POPS:POPC (5:3:2, wt/wt/wt) the most frequent conductance detected in our purified rat cerebral artery myocyte SR membrane preparation displayed a phenotype typical of homomeric RyR2, we next reconstituted recombinant homomeric RyR2 into POPE:POPS:POPC (5:3:2, wt/wt/wt) and set to determine whether RyR2 expressed in a simple lipid microenvironment was sensitive to intoxicating concentrations of EtOH.

Ion channels under study display a phenotype consistent with RyR2

Large DNA molecules are prone to deletions and/or rearrangements during plasmid propagation, a phenomenon that is particularly applicable to RyR2 cDNA-containing plasmids [32] (Wayne Chen, *personal communication*). On the other hand, reconstitution of RyR channels into phospholipid bilayers may incorporate ion channel proteins endogenous

to the cell membrane preparation in addition to the RyR protein of interest [33,34]. Therefore, it was critical to determine the RyR2 identity of the functional channel under study before evaluating its EtOH pharmacology. Following protein incorporation into a POPE:POPS:POPC (5:3:2, wt/wt), we detected well-resolved unitary current events in symmetrical 300 mM Cs^+ provided that the cytosolic side of the channel was exposed to Ca^{2+} levels≥1 μM (Fig. S1A). From gap-free recordings of no less than 3 min obtained at steady-voltages from −30 to 30 mV and Ca^{2+}≥1 μM, we obtained plots of NPo and unitary current amplitude (i) vs. voltage (Figs. S1B,C). A representative i/V plot demonstrates the ionic current's ohmic behavior within this voltage range (Fig. S1C), rendering a unitary slope conductance of 550±7 pS (n=9). According to Nernst's prediction for a channel highly permeable to Cs^+, the slope conductance changed to 187±5 pS in asymmetrical 300/50 mM (cis/trans) Cs^+. On the other hand, NPo remained relatively stable within −25 to +20 mV (Fig S1B), as reported for RyR2 [35] and rat cerebral artery myocyte native RyR in POPE:POPS:POPC (5:3:2, wt/wt/wt) bilayers, these native channels considered to result from homomeric association of RyR2 proteins [20].

One of the key phenotypic features of RyR is that NPo heavily depends on cytosolic Ca^{2+} levels [32,36]. Indeed, NPo of the channel under investigation was increased in response to Ca^{2+} chelation evoked by adding 0.9 mM EGTA to the bilayer *cis* chamber, which corresponded to the cytosolic side of the ion channel in our experimental setting (Fig. S2A). The treatment drops the nominal Ca^{2+} concentration from 1 mM to 100 μM (Maxchelator). At this level, the NPo-cytosolic Ca^{2+} relationship reaches near its maximum in native cerebral artery smooth muscle RyR, which are thought to consist of homomeric RyR2 [20].

Ryanodine binds and opens RyRs at a specific site leading to channel stabilization into a long-lived half-conductance state [37], a phenotype also replicated with rat cerebral artery myocyte native RyR [20]. Indeed, addition of 10 μM ryanodine to the bilayer cis solution shifted the channel under study from its main, short-lived full conductance of 550 pS to a long-lived subconductance state of 280 pS (Fig. S2A, **bottom trace**). Consistent with previous data from recombinant RyR2 expressed in lipid bilayers reporting that transitions to long-lived subconductances are highly infrequent in absence of ryanodine [32], we failed to detect such transition in absence of this alkaloid in 54 bilayers. Finally, the channel under investigation increased NPo in response to low mM levels of caffeine (Fig. S2B; free Ca^{2+} in *cis* solution ≤5 μM), as reported for all RyR types [38], including cerebral artery myocyte SR native RyR [20]. Collectively, unitary conductance and NPo behavior in response to changes in trans-bilayer voltage and different ligands label the unitary current events under study as mediated by RyR activity.

NPo was also monitored in 300/50 mM Cs^+ (*cis/trans*) and ≤5 μM Ca^{2+} in the *cis* solution at a holding potential of 10 mV before, during and after addition of 1 μM digoxin to the *cis* solution, a concentration that potentiates homomeric RyR2 but not other homomeric RyR isoforms [20]. Digoxin elevated NPo from a pre-digoxin value of 0.005 to 0.09, with NPo returning to pre-digoxin values following washout of the preparation in digoxin-free solution for 3 min (Fig. S2C). These data are identical to those from cardiac and cerebral artery smooth muscle SR native RyR, both thought to result from RyR2 tetrameric association [20,28]. In conclusion, the channel under study displays a phenotype typical of RyR2.

Ethanol reversibly inhibits RyR2

Following phenotypic channel identification, we also used single channel recordings from POPE:POPS:POPC (5:3:2, wt/wt/wt) bilayers to evaluate RyR2 response to short applications of 10–100 mM EtOH, that is, a pharmacological protocol similar to that used to probe Ca^{2+} sparks in the intact artery. In all bilayers probed with EtOH (n=39), three criteria were used to confirm the RyR2 phenotype of the channel under study: 1) unitary current amplitude corresponding to 187±5 pS in asymmetrical 300/50 mM (cis/trans) Cs^{+}; 2) observation of increase in NPo after dropping *cis* solution calcium levels from 1 mM to 100 μM. Only if channel behavior fulfilled criteria #1 and #2, we proceeded with EtOH probing. At the end of EtOH washout, we next: 3) confirmed the RyR phenotype by observing a shift of the channel main conductance to a long-lived subconductance state in response to 10 μM ryanodine at the cytoplasmic side of the channel (see Fig. S1A). Channels that did not fulfill these three criteria were not considered for analysis. As shown in Fig. 2A, RyR2 NPo was reversibly decreased by application of 50 mM EtOH to the *cis* side of the channel. Indeed, averaged NPo dropped to 52.5±2.8% of control during 50 mM EtOH and returned to 88.7±8.1% of pre-EtOH values after drug-free perfusion (Fig. 2B). Records from a bilayer containing a single RyR2 show that the reduction in RyR Po by 50 mM EtOH occurs in absence of noticeable changes in channel mean open times but is related to drug-induced increase in appearance of long closures (Fig. 2C), leading to lengthening of the channel mean closed time, i.e., decrease in the frequency of channel openings (see below). Indeed, exponential fitting of these data following published methods (see SI) renders the following mean open and mean closed times: control, 2.2 and 20.9 msec; 50 mM EtOH, 2 and 33.9 msec.

Ethanol-induced reduction of RyR2 NPo was consistently observed among bilayers made from different membrane preparations: NPo decreased in 4/4, 4/5, 4/4, 9/9 and 9/17 bilayers in presence of 3, 10, 18, 50 and 100 mM EtOH, respectively. Therefore, EtOH at clinically relevant concentrations reduced RyR2 NPo in 30/39 bilayers. Ethanol action on RyR2 NPo was concentration-dependent, with monotonic inhibition observed within 3–50 mM (Fig. 3A): EtOH levels corresponding to BAL that constitute legal intoxication in most US states (18 mM) decreased NPo to 62% of control, whereas EtOH levels corresponding to BAL following moderate-heavy episodic drinking (50 mM) decreased NPo to ~58% of control, practically reaching IC_{max}. However, 100 mM EtOH did not exert further inhibition (Fig. 3A). From a three-parameter sigmoidal fit to monotonic inhibition data, we obtained an IC_{50}=11.7 ± 1.9 mM (Fig. 3B), which is similar to that of Ca^{2+} spark inhibition. These data place RyR2 among the ion channel proteins that are most sensitive to EtOH.

The fact that EtOH action on RyR2 NPo reached a plateau at 50–75 mM to decrease (i.e., ameliorated inhibition) at 100 mM led us to consider whether an osmotic challenge to the bilayer by the higher EtOH concentrations could contribute to the reduced inhibition of NPo. Thus, we tested whether an osmotic effect could contribute to EtOH action on RyR2 by using urea to iso-osmotically substitute for EtOH in the bath solution. Urea and EtOH are both weak osmolytes, have a similar molecular weight and cross membranes rather similarly, which make urea a suitable compound to use as control for possible osmotic actions of EtOH [39]. Addition of 50 mM urea to the RyR2 cytosolic side, however, did not

reduce NPo (Fig. 4A top traces, Fig. 4B left bar), indicating that osmotic challenge *per se* does not participate in EtOH maximal inhibition of RyR2. In addition, 100 mM urea failed to significantly modify NPo, evoking a response similar to that triggered by 50 mM urea (Fig. 4A top traces, Fig. 4B left bar). Thus, while we cannot totally rule out some contribution of an osmotic effect-induced activation to the reduced inhibition of RyR2 in response to EtOH>50 mM, it is highly unlikely that an osmotic challenge *per se* explains such reduction, and additional mechanism(s) yet to be identified should be involved.

At all concentrations tested, EtOH action on RyR2 NPo occurred in absence of changes in unitary conductance: 187±5 vs. 187±4 pS in absence and presence of maximally inhibitory [EtOH] (50 mM). In absence of change in conductance and N (Discussion), EtOH action on RyR2 ionic current must be attributed to a decrease in Po, which can be explained by a decrease in RyR2 mean open time, an increase in mean closed time, or a combination of both. Within the range of EtOH concentrations that significantly reduced RyR2 NPo (10–50 mM), mean open times did not significantly differ from pre-EtOH values: 90±17, 76±3 and 89±14% of control (1.9±0.3 msec) in presence of 10, 18, and 50 mM EtOH, respectively ($P>0.05$). The presence of multi-channels bilayers of unknown N in all bilayers we examined in detail (n=39) prevented us from meaningful and systematic interpretation of closed-times distribution and determination of mean closed-times. However, a first glance at channel closed times can be obtained from evaluating the frequency of channel openings, which should change in inverse direction of closed time modifications. Frequency of RyR2 openings decreased to 75±12 ($P<0.02$), 56±10 ($P<0.05$) and 46±3% ($P<0.01$) of control (48.3±9.6/s) in presence of 10, 18, and 50 mM EtOH, respectively. These data suggest a concentration-dependent, EtOH-induced increase in RyR2 closed times, which is consistent with direct measurement of mean closed and open times obtained in absence and presence of 50 mM EtOH (Fig. 2C).

Collectively, our single channel data from artificial bilayers of simple phospholipid composition document that EtOH inhibition of RyR activity is independent of cytosolic second messengers, native membrane organization and complex proteo-lipid media, but likely results from a direct interaction between EtOH and RyR2 protein themselves and/or their immediate lipid environment that leads to stabilization of RyR closed state(s).

Discussion

Acute exposure to EtOH levels reached in blood during alcohol intoxication (≥18 mM) constricts cerebral arteries [7,8,9,10,14], an EtOH action linked to alcohol-related cerebrovascular disease [2,3,4,5,6,7]. Using rodent models, we previously documented that myocyte STOCs served as final effectors of EtOH concentration-dependent vasoconstriction, with EtOH direct inhibition of myocyte BK channels likely playing a significant role in EtOH-induced STOC inhibition [9,10,14]. In rat cerebral artery myocytes, however, STOCs occur due to Ca^{2+} sparks, a vasodilatory signal generated by SR RyR [13,15]. We now demonstrate that intoxicating EtOH levels that constrict cerebral arteries (10–50 mM) reduce both Ca^{2+} sparks in intact cerebral arteries (Fig. 1) and RyR2 NPo (Fig. 3), homomeric RyR2 being considered to constitute the major native RyR in rat cerebral myocytes [20].

EtOH inhibition of Ca^{2+} sparks and RyR2 was fully reversible and equipotent: EtOH IC_{50} (12–17 mM) places RyR2 among the very selective group of ion channels that i) alter activity in response to EtOH levels matching BAL that constitute legal intoxication in the US (10–18 mM; www.niaaa.org) and ii) mediate EtOH actions in the body (e.g., cerebral artery constriction) [40,41]. Reversibility, similar time-course, and equipotency of EtOH action on Ca^{2+} sparks and RyR2 NPo suggest a causal relationship between these phenomena. In an earlier study, we documented that 50 mM EtOH, which maximally decreases RyR2 NPo (Fig. 3), did not decrease but mildly elevated SR "Ca^{2+} load" [9]. Thus, EtOH inhibition of Ca^{2+} sparks must result primarily from drug-induced inhibition of RyR-mediated ionic current. The fact that EtOH-induced reduction of RyR2 NPo is reversible (Fig. 3A, B) strongly suggests that EtOH effect is not due to a decrease in N: it would be rather unlikely that EtOH washed away a fully functional RyR2 from the bilayer into the *cis* chamber solution and washout perfusion reincorporated the channel into the bilayer rendering back a fully-functional RyR2. In absence of changes in unitary conductance (see Results), EtOH action on RyR-mediated current is limited to that of a gating modifier, which results from drug-induced stabilization of channel closed states.

Ethanol alters RyR2 steady-state activity following recombinant channel protein reconstitution in an artificial membrane of controlled lipid composition, which leads us to hypothesize that EtOH action results from a direct interaction between the drug and RyR2 proteins themselves and/or their immediate proteo-lipid microenvironment. The simple chemical composition and thus organization of the artificial bilayer used in our study argues against involvement of complex membrane architecture and domains in EtOH action. RyR2 reconstituted into bilayers, however, associates with a variety of regulatory proteins, FKB12.6 in particular [42,43]. While we cannot totally rule out the involvement of FKB12.6 in present results, previous studies documented that the HEK293 preparation expressing RyR2 used in our study contained negligible amount of FKB12.6, if any [44]. Finally, the fact that our solutions contained no Mg^{2+}, nucleotides or other "regenerating" systems, and that EtOH action was evaluated several minutes after RyR2 incorporation under continuous bath perfusion, make it very unlikely that most kinases, freely diffusible cytosolic messengers, and cell organelles play a role in EtOH inhibition of RyR2.

In contrast to present results from RyR2, the frog skeletal muscle RyR has been reported to be insensitive to 2.2–217 mM EtOH [26,45]. A plausible explanation for the discrepancy between these findings and ours resides in RyR isoforms themselves, as skeletal muscle RyR are of type 1. Other explanations reside in putative differences in proteo-lipid composition and/or redox state associated to RyR2. Interestingly, RyR2 inhibition by 100 mM EtOH was observed in only 55% of bilayers, which contrasts with the highly consistent inhibition (95% of bilayers) observed at lower EtOH levels (3–50 mM). The mechanisms underlying this heterogeneity remain speculation. Since our data were obtained with recombinant protein showing a homogenous phenotype distinct of RyR2 following reconstitution in a bilayer of controlled lipid composition, it is highly unlikely that heterogeneity channel response to different EtOH levels (3–50 vs. 100 mM) is due to RyR subunit heterogeneity or complex lipid organization. Conceivably, as reported for other

Ca^{2+}-gated ion channels [40,46], high EtOH levels could favor ligand-driven RyR desensitization.

In conclusion, we identified RyR2 as a novel, highly-sensitive target for clinically relevant EtOH concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EtOH	ethanol
BK	Ca^{2+}- and voltage-gated K^+
STOC	spontaneous transient outward current
SR	sarcoplasmic reticulum
RyR	ryanodine receptor
PSS	physiological saline solution
POPE	1-palmitoyl-2-oleoyl-phosphatidylethanolamine
POPS	1-palmitoyl-2-oleoyl-phosphatidylserine
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
N	number of channels present in the lipid bilayer
Po	individual channel open probability
BAL	blood alcohol level

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Highlights

- We studied ethanol action on RyR2 in cerebral arteries and isolated receptors
- Ethanol blunted RyR-mediated Ca^{2+} spark frequency in arteries with IC_{50}=17 mM
- Ethanol inhibited RyR2 reconstituted into phospholipid bilayers with IC_{50}=12 mM
- Our study places RyR2 among the ion channels that are most sensitive to ethanol
- Ethanol action on RyR2 may contribute to alcohol-induced artery constriction

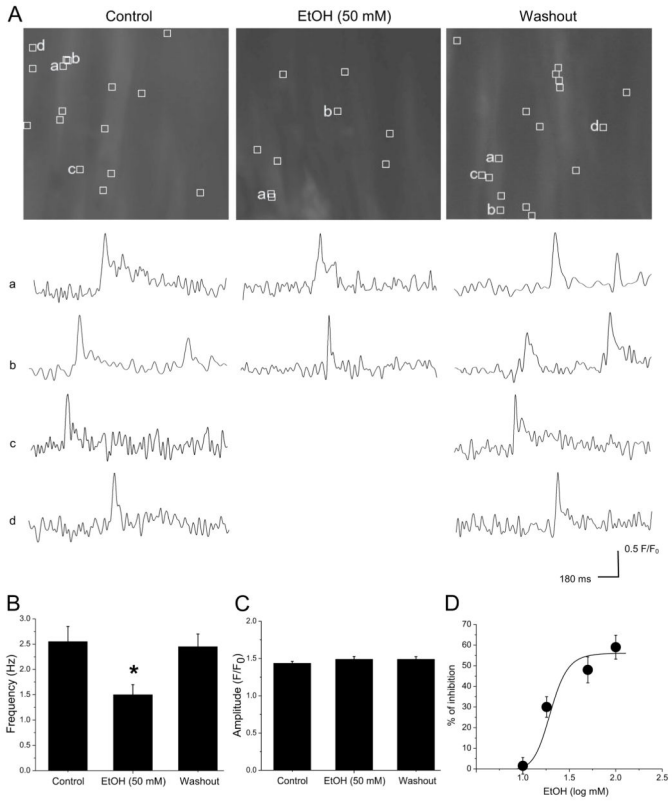

Fig. 1.

Ethanol at clinically relevant concentrations reversibly inhibits Ca^{2+} sparks in rat cerebral artery. (A) Representative spark images and traces representing Ca^{2+} sparks in cerebral artery before (control), during (EtOH) and after (washout) EtOH application. Bar graphs showing significant decrease in Ca^{2+} spark frequency (B), but not amplitude (C) in presence of 50 mM EtOH. **P*<0.05 vs. control. (D) Concentration-response curve of EtOH-induced inhibition of Ca^{2+} sparks. In (B–D) each data point was obtained from averaging data from no less than 3 arteries (10 mM, n=5; 18 mM, n=5; 50 mM, n=4; 75 mM, n=3).

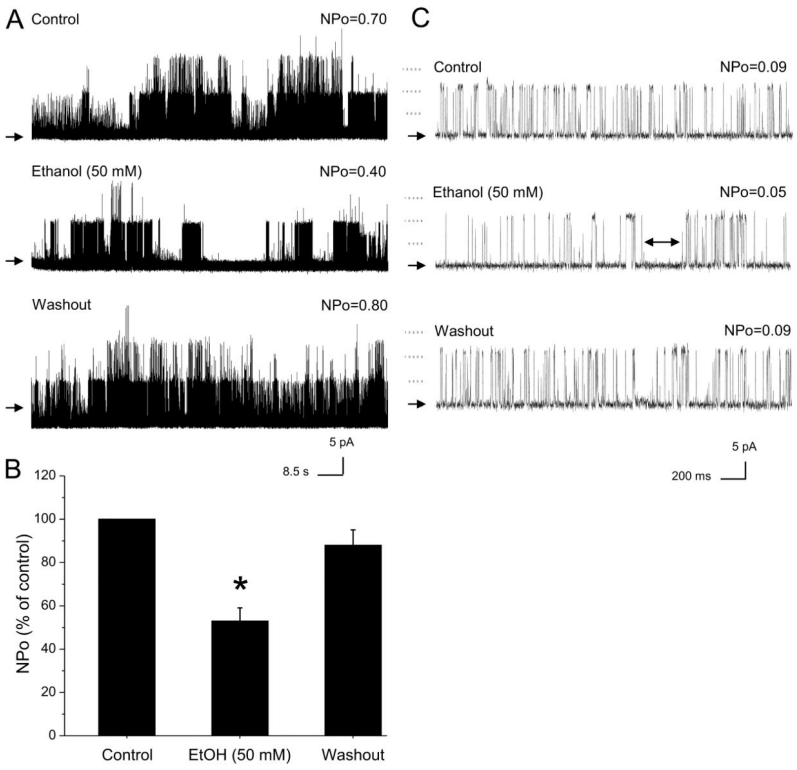

Fig. 2.

EtOH reversibly inhibits recombinant RyR2 channels reconstituted into planar lipid bilayers primarily by lengthening the channel mean closed time. (A) Records showing RyR2 channel activity before (control), during (EtOH), and after application of 50 mM EtOH. (B) Averaged data showing significant and reversible decrease in RyR NPo by 50 mM EtOH. **P*<0.05 vs. control. (C) Single channel records from a bilayer containing a single RyR (N=1) showing that EtOH-induced reduction in Po is associated with a significant lengthening of the channel closed times. In the middle trace, a double-headed arrow underscores the type of long closures that were introduced by EtOH while being more infrequent in absence of the drug. Indeed, exponential fitting of these data following previously published methods (see SI) renders the following mean open and mean closed times: control, 2.2 and 20.9 msec; 50 mM EtOH, 2 and 33.9 msec. Data were acquired with free Ca^{2+} in the *cis* solution set to 100 μM.

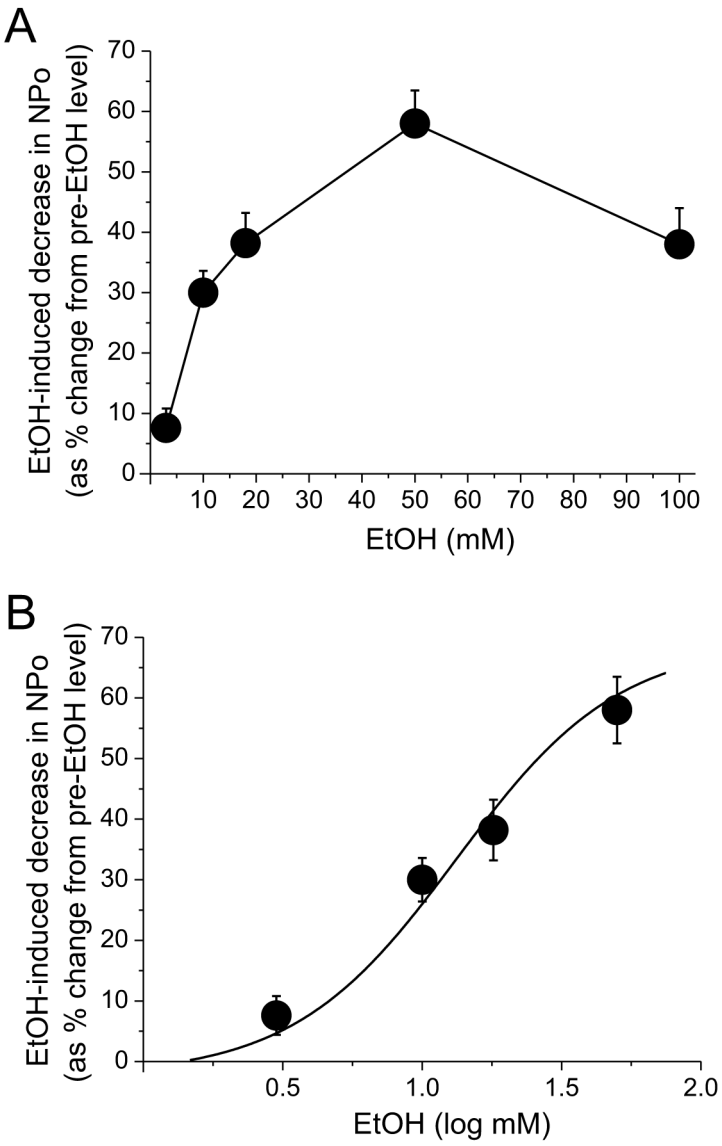

Fig. 3. Ethanol-induced inhibition of RyR2 activity (NPo) is concentration-dependent, reaching maximal inhibition at 50 mM

(A) Plot of RyR NPo as function of [EtOH]. (B) Concentration-response-curve of EtOH action, plotted as percentual change in NPo from pre-EtOH (control) values. Data were acquired with free Ca^{2+} in the *cis* solution set to 100 μM. In (A–B) each data point was obtained from averaging data from no less than 4 bilayers (3 mM, n=4; 10 mM, n=4; 18 mM, n=4; 50 mM, n=9; 75 mM, n=9).

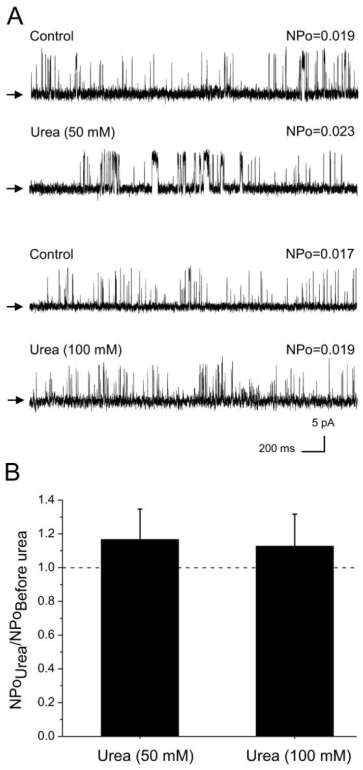

Fig. 4.

Lack of response in RyR activity to osmotic challenge. (A) Representative records showing RyR activity before (control) and during application of 50–100 mM urea. Arrows point at the baseline (all channels closed). (B) Bar graph showing RyR activity in presence of urea compared to control. Dotted line defines lack of effect by urea. All data were acquired with free Ca^{2+} in the *cis* solution set to 100 μM. Averaged data from 50 and 100 mM urea with corresponding controls were obtained from 3 and 4 bilayers, respectively.