FEBS Letters 588 (2014) 1659–1665

journal homepage: www.FEBSLetters.org

Type 2 ryanodine receptors are highly sensitive to alcohol

Yanping Ye [a], Kuihuan Jian [a], Jonathan H. Jaggar [b], Anna N. Bukiya [a], Alex M. Dopico [a,*]

[a] Department of Pharmacology, The University of Tennessee Health Science Center, Memphis, TN 38163, United States
[b] Department of Physiology, The University of Tennessee Health Science Center, Memphis, TN 38163, United States

ARTICLE INFO

Article history:
Received 28 January 2014
Revised 27 February 2014
Accepted 1 March 2014
Available online 12 March 2014

Edited by Maurice Montal

Keywords:
Ryanodine receptor
Alcohol
Cerebral artery smooth muscle
Lipid bilayer electrophysiology

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.

1. Introduction

Moderate to heavy episodic alcohol intake is associated with an increased risk for cerebral artery constriction and disease [1–4]. Alcohol-induced cerebrovascular disease and modification of vascular physiology is independent of beverage type but linked to ethanol (EtOH) itself [1,5–7]. Acute exposure to EtOH levels reached in circulation during alcohol intoxication (10–100 mM) constricts cerebral arteries in several species, including rats and humans [7–11]. 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 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 betal 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: RyRl, RyR2 and RyR3 [17–19]. 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.

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, l-palmitoyl-2-oleoyl-phosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; POPC, l-palmitoyl-2-oleoyl-phosphatidylcholine; N, number of channels present in the lipid bilayer; Po, individual channel open probability; BAL, blood alcohol level

* Corresponding author. Address: 874 Union Ave., Memphis, TN 38163, United States. Fax: +1 (901)448 2260.
E-mail address: adopico@uthsc.edu (A.M. Dopico).

http://dx.doi.org/10.1016/j.febslet.2014.03.005
0014-5793/

2. Materials and methods

2.1. 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].

2.2. 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 −80 °C.

2.3. 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.

2.4. 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.

3. Results

3.1. 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 [1–4,7,8], 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/by-products 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 [28–30], (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.

3.2. 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 ⩾ l 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 (Fig. 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 infre-