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Research report

Dantrolene blockade of ryanodine receptor impairs ethanol-induced behavioral stimulation, ethanol intake and loss of righting reflex

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HIGHLIGHTS

- Dantrolene reduction of ethanol-induced motor stimulation, ethanol intake and LORR.
- Dantrolene selectively modulates the neurobehavioral effects of ethanol.
- Ca^{2+} release through RyR mediates ethanol behavioral effects.

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ABSTRACT

Calcium has been characterized as one of the most ubiquitous, universal and versatile intracellular signals. Among other substances with the ability to alter intracellular calcium levels, ethanol has been described as particularly relevant because of its social and economic impact. Ethanol effects on calcium distribution and flux *in vitro* have been widely studied, showing that acute ethanol administration can modulate intracellular calcium concentrations in a dose dependent manner. Intracellular calcium released from the endoplasmic reticulum plays a determinant role in several cellular processes. In this study, we aim to assess the effect of dantrolene, a ryanodine receptor antagonist, on three different ethanol-elicited behaviors: locomotor activity, loss of righting reflex and ethanol intake. Mice were challenged with an injection of dantrolene (0–5 mg/kg, i.p.) 30 min before ethanol (0–4 g/kg, i.p.) administration. Animals were immediately placed in an open field cylinder to monitor distance travelled horizontally or in a V-shaped trough to measure righting reflex recovery time. For ethanol intake, dantrolene (0–5 mg/kg, i.p.) was administered 30 min before ethanol (20%, v/v) exposure, following a drinking in the dark paradigm. Our results showed that dantrolene selectively reduces ethanol-induced stimulation, loss of righting reflex, and ethanol intake in a dose dependent manner. Together, these data suggest that intracellular calcium released from the endoplasmic reticulum may play a critical role in behavioral effects caused by ethanol, and point to a calcium-dependent pathway as a possible cellular mechanism of action for ethanol.

1. Introduction

Ethanol (EtOH) is known to be one of the most widely consumed substances of abuse, with a deep social and economic impact. Although it has dramatic effects on the central nervous system (CNS), research to date has failed to identify a unique molecular mechanism by which this alcohol alters cellular function [1,2]. Nevertheless, acute behavioral effects of EtOH are observed within short periods of time, suggesting an immediate interaction of EtOH with some receptors and/or excitable membrane proteins in the CNS [3–6]. In addition, it is known that EtOH alters the function of specific membrane-bound gated ion channels [7–10] as well as the flux dynamics of several ions through the cytiplasmic membrane and different cell organelles [1,6,8,10].

It has been described that calcium (Ca^{2+}) contributes to neuronal excitability *via* its role in action potential dynamics [11,12] and also that Ca^{2+} plays an important role in several brain functions, such as neurotransmitter release, second messenger cascades, several forms of plasticity, and neuronal death and survival [13,14]. In this sense, cells can generate these Ca^{2+} signals by using internal, external or combination of both sources of Ca^{2+}. Within the CNS, the internal stores are held within the membrane system of the endoplasmic reticulum (ER) or by other organelles such as mitochondria and a variety of acidic compartments [15–17].

Acute EtOH exposure in cell cultures has been proved to modulate Ca^{2+} flux [18–21]. In addition, it is generally accepted that physiologically relevant EtOH concentrations (<80 mM) increase

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intracellular Ca^{2+} inward currents in different cell cultures such as hippocampal neurons [22], superior cervical ganglion neurons [6], and brain microsomes [23]. Moreover, some authors have found that this EtOH-induced increase in Ca^{2+} inward currents may be triggered by Ca^{2+} release from different cellular internal stores, with the role of ER being of special interest [19,24–28]. As commented above, effects of ethanol on intracellular Ca^{2+} dynamics have been widely studied in different cell preparations, but not at the behavioral level [29–34]. In this respect, recently published data from our laboratory, suggested the central actions of ethanol require normal distribution and flux of intracellular Ca^{2+} [35]. In fact, Ca^{2+} release from the ER is controlled following a concrete temporal pattern by various channels of which the inositol-1,4,5-trisphosphate receptor (InsP3R) and ryanodine receptor (RyR) families have been studied most extensively [36–38]. In this sense, compared to the cellular responses involving EtOH interacting with InsP3R-mediated pathways such PKC [39–42], little is known about the participation of the RyR Ca^{2+} currents in mediating some of the cellular and behavioral effects of EtOH. The RyR is one of the main channels through which ER releases Ca^{2+} within the cell, which makes it a critical structure in cellular processes involving intracellular Ca^{2+} [43–46]. Within the pharmacological tools for manipulation of ER receptors described in the literature, dantrolene remains the only drug targeting RyR channels to be used clinically [44,47]. Dantrolene has been tested primarily in skeletal muscle [48], but its actions on Ca^{2+} release extend also to other type of cells, including neurons [49–52]. This compound has been used in behavioral procedures [53] demonstrating that intracerebroventricular administration of dantrolene inhibited the development of cocaine-induced place preference in mice. In this study, we aimed to assess for the first time the effect of RyR blockade on different EtOH-elicited behavioral responses such as EtOH-induced locomotor activity, EtOH intake and EtOH sedative effects in mice. The aims of this investigation were also extended to study whether RyR blockade is selective to the effects of EtOH when compared to other drugs of abuse such as amphetamine and cocaine.

2. Methods

2.1. Animals and housing

Two different strains of mice were used in this work. A total of 9–12 male CD-I mice (Harlan, Interfauna) per group, aged 6–8 weeks, were used in locomotor activity and loss of righting reflex (LORR) studies. Swiss CD-I mice used in this study were chosen for its steady response to the stimulating and hypnotic effects of ethanol [35,54,55]. For the EtOH or sucrose intake experiments, 16 male C57BL/6J (B6) mice (Charles River Laboratories, Barcelona, Spain) per group aged 6–8 weeks were used. This strain of mice was used in this study because of their genetic background of high EtOH preference [56,57].

All mice, aged 4–6 weeks upon arrival, were housed 3 per cage in an acclimated quarantine room in which they remained for a week, except for the B6, which were allocated individually. Animals were moved into a colony room at least one week before the experiments started. The colony room was maintained at a temperature of $21 \pm 1\,^{\circ}C$ and controlled under a 12-h light/dark cycle (lights on at 08:00 h) for the locomotor activity and LORR experiments. EtOH intake room was prepared as described following Rhodes et al. [56]. Food and water were provided *ad libitum* throughout the study. All experimental procedures complied with the European Community Council Directive (86/609/ECC).

2.2. Drugs

EtOH, purchased from Panreac (Barcelona, Spain) and used for intraperitoneal (i.p.) injections, was diluted to 20% (v/v) in 0.9% (w/v) physiological saline. For the voluntary intake experiments, EtOH (20%, v/v) and saccharin (0.1%, w/v) (Sigma–Aldrich Química, Madrid, Spain) were diluted in tap water. Dantrolene sodium, cocaine, amphetamine, and pentobarbital were purchased from Sigma–Aldrich Química (Madrid, Spain). All solutions were diluted in 0.9% (w/v) physiological saline and prepared the same day of the experiment.

2.3. Behavioral procedures

To address the role of Ca^{2+} flux through RyR on EtOH-elicited behaviors such locomotor activity, LORR and EtOH intake, we have divided this work in three experimental phases with regard to the different paradigms studied here.

The first set of experiments explored the effects of different doses of dantrolene on the stimulating effects of EtOH. Animals ($n = 9$–12 per group) were moved in their home cages to the testing room 30 min before starting. Experiment 1 assessed the effect of dantrolene (0, 0.625, 1.25, 2.5 or 5 mg/kg, i.p.) on EtOH-induced locomotor activity. The previous dantrolene doses were chosen based on different *in vivo* studies that demonstrated a blocking effect of RyR within the same dose range [58,59]. Mice were injected with saline or one of the four doses of this RyR blocker and returned to their home cages for a waiting period of 30 min. After this time, an i.p. challenge dose of 0 or 2.5 g/kg of EtOH was administered immediately prior to placing animals in the open field where horizontal activity was measured for 20 min. Experiment 2 studied the effects of several EtOH doses (0, 1.5, 2.5 and 3.5 g/kg, i.p.) on locomotion in animals pretreated with dantrolene (0 or 1.25 mg/kg, i.p.). This EtOH dose range was chosen in order to evaluate the effect of dantrolene on the stimulant-to-sedative effects of EtOH [54,60]. A third experiment was conducted to explore the temporal pattern of the effect of dantrolene pretreatment on EtOH-induced stimulation. Animals were pretreated with dantrolene (0 or 1.25 mg/kg, i.p.) 0, 15, 30, 60 and 120 min before EtOH (2.5 g/kg, i.p.). Experiment 4 explored the effects of dantrolene on locomotor stimulation induced by the following drugs: amphetamine and cocaine. With this experiment we assessed whether the antagonism of RyR found in previous experimental phases of EtOH stimulation is shared by other drugs with psychomotor properties. Mice received amphetamine (0 or 2 mg/kg, i.p.) or cocaine (0 or 4 mg/kg, i.p.), 30 min after saline and dantrolene (5, 10 and 20 mg/kg, i.p.). Immediately after drug challenge, locomotor activity was measured following the same conditions described for EtOH. Doses of amphetamine and cocaine were selected in accordance with previous studies [54,61,62].

For all experiments, testing took place between 10:00 h and 14:00 h, after which they were placed back in their home cage.

In a second set of experiments, we addressed the effects of dantrolene and pentobarbital administration on EtOH induced LORR. We followed a modified version of a previously described method [55,63]. Briefly, dantrolene (0 or 1.25 mg/kg; i.p.) was administered 30 min prior to an EtOH (4 g/kg; i.p.) or pentobarbital (50 mg/kg; i.p.) injection able to induce LORR. In mice, this concentration yields LORR durations ranging from 30 to 120 min, and covers the range of doses between the minimum necessary to produce the response and the maximum that will not cause death. We have contemplated two variables: latency and duration of the LORR effect. LORR latency was defined as the time immediately following injection during which the animal was unable to right itself onto all four paws. The duration of LORR was defined as the time from loss of the righting reflex to the time of recovery of the righting reflex (ability to right three times in 60 s).

The third set of experiments addressed the effect of dantrolene administration on EtOH intake. As we have commented above, we performed a variant of the Drinking-In-The-Dark (DID) procedure [56]. Briefly, water tubes were replaced with 10 ml graduated cylinders with sipper tubes containing 20% EtOH or 0.1% saccharin 3 h after the dark cycle started. During the 4-day phase (Day 1–Day 4), EtOH/saccharin cylinders were available for 2 h and then replaced with water bottles. Animals were left undisturbed for two days when these four days finished. Following this phase, four 2-day sessions were conducted. In accordance with a within-subjects design, the 2-day DID procedure was repeated twice a week for two weeks. On the second day of each 2-day session, each mouse received either an i.p injection of saline or 1.25, 2.5 or 5 mg/kg of dantrolene 30 min before water bottles were replaced for 2 h with EtOH/Saccharin. The order in which the four doses of dantrolene were administered was counterbalanced across animals. On the first day of each 2-day session, animals did not receive any injections. Thus, before the test day, they always had 1 day of access to the drinking solution without injections, and 1 or 2 days where they were left undisturbed. The aim of allowing the mice to have access to drinking solution for one day without any injections is to reduce the possible development of taste aversion from pairing EtOH/saccharin exposure with an injection.

2.4. Apparatus

2.4.1. Locomotor activity test

For the locomotor activity experiments, all subjects were tested in open-field chambers (25 cm-diameter and 30 cm-high dull glass cylinders), placed in an attenuated sound and light procedure room (8 m^2; 20 W, regular white light) to minimize stress induced by highly illuminated environments. Locomotor activity was registered by a computerized video-tracking system (S.M.A.R.T., Panlab, Barcelona, Spain) and later translated by Ethovision software into horizontal distance traveled (cm). The duration of the test was 20 min.

2.4.2. Loss of righting reflex

For the LORR test, animals were placed in classic V-shaped troughs and assessed for LORR. The experimental room was sound and light attenuated (8 m^2; 20 W, regular white light) in order to minimize stress induced by highly illuminated environments. After the mice lost the righting reflex, they were put on their backs in their home cage. The duration of LORR was defined as the time from the loss of the righting reflex to the time at which it was regained. Animals were judged to have regained their righting reflex when they could right themselves three times within 1 min after being placed on their backs.