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ORIGINAL INVESTIGATION

Role of CA2+/calmodulin on ethanol neurobehavioral effects

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Abstract
Rationale The cAMP-dependent protein kinase A (PKA) signaling transduction pathway has been shown to play an important role in the modulation of several ethanol-induced behaviors. Different studies have demonstrated intracellular calcium (Ca^{2+})-dependent activation of the PKA cascade after ethanol administration. Thus, the cAMP cascade mediator Ca^{2+}-dependent calmodulin (CaM) has been strongly implicated in the central effects of ethanol.
Objectives In this study, we assessed the role of the CaM inhibitor W7 on ethanol-induced stimulation, ethanol intake, and ethanol-induced activation of PKA.
Methods Swiss mice were pretreated with W7 (0–10 mg/kg) 30 min before ethanol (0–3.75 g/kg) administration. Immediately, animals were placed during 20 min in an open-field chamber. Ethanol (10 %, *v*/*v*) intake in 2 h was assessed using a limited access paradigm. Experiments with caffeine (0–15 mg/kg), cocaine (0–4 mg/kg), and saccharine (0.1 %, *w*/*v*) were designed to compare their results to those obtained with ethanol. Western blot was assayed 45 min after ethanol administration.
Results Results showed that pretreatment with W7, reduced selectively in a dose-dependent fashion ethanol-induced locomotor stimulation and ethanol intake. The ethanol-induced activation of PKA was also prevented by W7 administration.
Conclusions These results demonstrate that CaM inhibition resulted in a selective reduction of ethanol-stimulating effects and ethanol intake. The PKA activation induced by ethanol was blocked after the CaM blockade with W7. These results provide further evidence of the key role of cellular Ca^{2+}-dependent pathways on the central effects of ethanol.

Keywords Ethanol · Locomotor stimulation · Ethanol intake · Calmodulin

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Introduction

Alcohol exerts a wide range of effects through its interaction with several neurotransmitters and neuromodulators in the CNS. Behavioral consequences observed after ethanol administration have been well characterized; however, the molecular mechanisms by which ethanol alters cellular function and how some of these alterations translate to changes in behavior are still unclear. To date, research has failed to identify a unique mechanism of action for alcohol in the CNS. The cAMP signaling pathway has emerged as an important modulator of ethanol sensitivity. Thus, in vivo, it has been demonstrated that manipulations of the cAMP signaling, affected different behavioral responses elicited by ethanol (Lee and Messing 2008; Maas et al. 2005; Wand et al. 2001). Recent published data from our laboratory demonstrated intracellular calcium (Ca^{2+})-dependent activation of the cAMP-dependent protein kinase (PKA) cascade after ethanol administration (Baliño et al. 2013). At this respect, the PKA signaling transduction pathway has been shown to play an important role in the modulation of several ethanol-induced behavioral actions. Manipulations involving the cAMP/PKA pathway modulate the ethanol-induced locomotor activity and sensitization (Freund and Palmer 1997; Hayes et al. 2012; Tolliver et al. 1999), ethanol sensitivity (Thiele et al. 2000; Wand et al. 2001), and ethanol intake (Pandey et al. 2005).

The cAMP synthesis is determined by the activity of the adenylyl cyclases (AC), which convert ATP to cAMP. Different AC subtypes have been found to be highly expressed in the mammal brain and have also been suggested as critical mediators of the cAMP/PKA cascade (Conti et al. 2009). Among others, it has been shown that AC1 and AC8 play a critical role in mediating the cAMP-dependent signal transduction in response to ethanol (Conti et al. 2009; Maas et al. 2005; Wand et al. 2001). Moreover, AC1 and AC8 are directly stimulated by Ca^{2+} via calmodulin (CaM) activation (Cali et al. 1994; Wang et al. 2003).

CaM has been described as one of the key proteins on transducing a signal in response to intracellular Ca^{2+}increase. It serves as a ubiquitous intracellular Ca^{2+}receptor (Chin and Means 2000) and is expressed in all eukaryotic cells where it participates in different signaling pathways that regulate many crucial cellular processes. CaM is a 148-amino acid protein (16,680 daltons) that comprised four helix–loop–helix protein folding motifs called EF hands, with two making up the N-terminal domain and two comprising the C-terminal domain. Upon calcium binding, CaM activates members of a family of serine/treonine protein kinases called Ca^{2+}/CaM-dependent protein kinases such as CaM kinases I, II, and IV and myosin light-chain kinase (Hook and Means 2001). These kinases have been involved in many aspects of neuronal function, including synaptic plasticity, gene expression, and neurotransmitter synthesis and release. Furthermore, Ca^{2+}/CaM kinases have been described as an important molecular mechanism involved in the neurobiological response to drug abuse (Jackson et al. 2012; Robison et al. 2013). At this respect, different Ca^{2+}/CaM kinases such CaM kinase II, have been described to play a key role on the behavioral response to ethanol in a rodent model (Easton et al. 2013; Wang et al. 2012). However, the particular mechanism by which ethanol modulates the activity of the Ca^{2+}/CaM kinases has not been clarified in detail yet.

Interestingly, ethanol administration has proved to modulate Ca^{2+}distribution and flux in different neuronal cell preparations (Cofán et al. 2000; De Beun et al. 1996). In addition, acute exposure to ethanol increases intracellular Ca^{2+}in a dose-dependent manner when administered at concentrations (<80 mM) and decreases at greater concentrations (>120 mM) (Belia et al. 1995). In fact, studies using brain synaptosomes and cultures of rat hippocampal neurons showed that an acute administration of ethanol produced an increase in intracellular Ca^{2+}, including medium solutions containing zero Ca^{2+}, as a result of its release from internal stores (Daniell and Harris 1989; Mironov and Hermann 1996).

Given this, the aim of the present investigation was to evaluate the role of Ca^{2+}/CaM on ethanol-induced stimulation and ethanol intake. Thus, we have selected the CaM blocker W7. This compound binds with high affinity to CaM in the presence of Ca^{2+}, and thereby blocks access to the activation site exposed when the Ca^{2+}-binding sites in CaM are occupied (Osawa et al. 1998; Qu et al. 2007). We hypothesized that the CaM blockade by the W7 agent would decrease the cAMP/PKA pathway activity and therefore ethanol-induced stimulation and ethanol intake. The aims of this investigation were also extended to study whether this pharmacological tool is selective in reducing ethanol-stimulating effects or ethanol intake when compared with other drugs with motor-stimulating properties and to a sweetened solution. The results derived from the present work would support a new ethanol mechanism of action at a central level that involves intracellular Ca^{2+} release to further activate a cAMP/PKA-dependent cascade through a CaM-dependent mechanism.

Materials and methods

Animal husbandry

Naive male Swiss (CD-I) mice, purchased from Elevage Janvier (Saint-Genest, France) were used in this study. Mice aged 4 weeks upon arrival at our laboratory were housed (three per cage) and acclimated to the colony room at least 2 weeks prior to the initiation of the study. Room temperature was maintained at 22±1 °C and humidity (50 %) in a 12/12-h light–dark cycle (lights on at 8 a.m.). Animals had free access to rodent chow (Panlab, Barcelona, Spain) and tap water. Swiss CD-I mice used in this study were chosen for its steady response to the stimulating and hypnotic effects of ethanol (Correa et al. 2001; Pastor et al. 2002). This CD-I mice strain was also used for the immunoblot analysis. For the ethanol intake experiment, 16 male C57BL/6 J (B6) mice (this JAX® mice strain was purchased from Charles River Laboratories España S.A., Barcelona) per group aged 6–8 weeks were used. Mice were housed four per cage for the first 2 weeks, and then transferred to individual cages for 1 week prior to the beginning of the experiment. Animals had free access to rodent chow (Panlab, Barcelona, Spain) and tap water. Room temperature was maintained at 22±1 °C and humidity (50 %) in a 12/12-h light–dark cycle (lights off at 7 a.m.). Red incandescent lamps were kept on during the dark phase so that investigators could handle mice. This strain of mice was used in this study because of their genetic background of high ethanol preference (McLearn and Rodgers 1959; Rhodes et al. 2005).

All experimental procedures complied with the European Community Council Directive (86/609/ECC).

Drugs and drinking solutions

Ethanol (Panreac, Barcelona, Spain) stocked at 96 % in a glass container, was diluted to 20 % (*v*/*v*) in 0.9 % (*w*/*v*) physiological saline for the injections (saline solution was administered to control groups). Ethanol (2–10 %, *v*/*v*) and saccharine (0.1 %, *w*/*v*) for drinking solution was diluted to the appropriate

concentration in tap water. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), caffeine, and cocaine were purchased from Sigma-Aldrich Química (Madrid, Spain); all doses of these compounds (see "Procedure") were diluted in 0.9 % physiological saline in an injection volume of 10 ml/kg. All solutions were administered intraperitoneally (i.p.) and prepared fresh daily in saline.

Procedure

Locomotor stimulation induced by ethanol

Seven different independent experimental phases were conducted to assess the effects of CaM blockade with W7 on ethanol neurobehavioral effects in mice. Animals (n=12–15) were moved from their home cages to the locomotor activity testing room at least 30 min before experiment initiation to allow acclimation to environmental conditions. Testing occurred between 10 a.m. and 4 p.m. Experiment 1 assessed the effect of W7 (0, 1.25, 2.5, 5, and 10 mg/kg, i.p.) on ethanol-induced locomotor activity. These doses were used in accordance with previous published work (Barron et al. 1986; Ito 1986). Moreover, there is evidence suggesting that W7 can be administered systemically at doses (0–10 mg/kg) that would have no effect on mice spontaneous behavior and has direct CNS actions (Barron et al. 1986; Ito 1986; Osawa et al. 1998). Mice (n=12–15) were injected with saline or one of the four doses of W7 and returned to their home cages for a waiting period of 30 min. After this time, a challenge dose of ethanol (0 or 2.5 g/kg, i.p.) 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 ethanol doses (0, 1.25, 2.5, and 3.75 g/kg, i.p.) on locomotion in animals (n=12–15) pretreated with W7 (0 or 5 mg/kg, i.p.). This ethanol dose range was designed to evaluate the W7 role from the stimulant-to-sedative effects of ethanol (Pastor et al. 2002; Pohorecky 1977). Experiment 3 was conducted to explore the temporal pattern of the effect of W7 on ethanol-induced stimulation. Animals (n=12–15) were pretreated with W7 (0 or 5 mg/kg, i.p.) 15, 30, or 60 min before ethanol (2.5 g/kg, i.p.). Experiment 4 explored the effects of W7 on locomotor stimulation induced by the following drugs: caffeine and cocaine. Here, we have evaluated whether the effects of W7 found in previous experimental phases on ethanol stimulation are shared by other drugs with psychomotor properties. Mice (n=10–12) were injected with W7 (0, 2.5, 5, and 10 mg/kg, i.p.) 30 min before caffeine (15 mg/kg, i.p.) or cocaine (4 mg/kg, i.p.) administration. Animals were placed immediately in the open field for 20 min to measure the locomotor activity. Doses of caffeine and cocaine were selected in accordance with previous studies that have demonstrated a locomotor activity increase on in our experimental conditions (Font et al. 2001; Pastor et al. 2002).

Ethanol intake

The methodology followed in the drinking procedures corresponds to a limited-access paradigm. Briefly, alcohol was always presented at the beginning of the third hour of the animals' dark cycle (at 10 a.m.). Prior to the initiation of the experiments, mice were subjected to a habituation period. Every 2 days, mice received escalating ethanol concentrations, from 2 to 10 % (*v*/*v*) during ten consecutive days. This period of habituation to ethanol drinking has previously been used by other authors and serves to accustom animals to the smell and flavor of ethanol (Wise 1973, 1975). It also avoids the neophobia initially induced by the presentation of new fluids in rodents (Aragon and Amit 1993; Correa et al. 2004; Font et al. 2001). During ethanol presentation, the water bottles were replaced with 10 ml graduated cylinders containing an ethanol solution for 2 h. When a 10 % concentration of ethanol was achieved, the baseline phase started, which consists in presenting a 10 % (*v*/*v*) solution until the intake of all mice reached stable levels during at least 5 days. Following this habituation period, four 2-day test sessions were conducted. In accordance with a within-subjects design, these sessions were repeated twice a week for 2 weeks. Experiment 5 assessed the effects of several doses of W7 on ethanol intake. On the first test day, all mice (n=16) received saline injections prior (30 min) to alcohol presentation. 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 W7 at the same time interval as on the preceding day (30 min before water bottles were replaced for 2 h with ethanol). The order in which the four doses of W7 were administered was counterbalanced across animals and days. In experiment 6, we have evaluated the effect of our pharmacological manipulations on voluntary saccharin intake. The same experimental design was repeated with a new cohort of animals (n=16), with the exception that in this case, no habituation period was needed to obtain stable saccharin (0.1 %, *w*/*v*) intake levels. The volume (ml) of ethanol or saccharine consumed were recorded 30 min each during 2 h. Ethanol consumed was later translated into grams per kilogram.

Determination of blood ethanol levels

To study whether the CaM blocker W7 used in the present experiments altered blood ethanol levels at the time of testing, a separate experiment with no behavioral testing involved was conducted. Experiment 7 evaluated the ethanol pharmacokinetic response to W7. Animals (n=6) were pretreated with W7 (0 or 5 mg/kg, i.p.) 30 min before ethanol treatment (2.5 g/kg, i.p.). Tail-vein blood samples (20 μl) were collected 10, 20, 60, and 120 min after ethanol administration. Each blood sample was immediately placed in a microcentrifuge tube containing 50 μl of ice-cold 5 % $ZnSO_4$ solution. A 50-μl

aliquot of 0.3 N Ba(OH)2 and 300 μl of deionized water were added. After centrifugation at 4 °C (5 min, 12,000 rpm), the supernatant was removed and blood ethanol levels were determined using headspace gas chromatography (CE Instruments GC 8000, HS 850) (Boehm et al. 2000).

Protein immunoblot analysis

Experiment 8 evaluated the effects of W7 administration on ethanol-induced PKA activity in different brain areas. Animals (n=5–10) were pretreated with W7 (0 or 5 mg/kg, i.p.) 30 min before an ethanol challenge (0 and 2.5 g/kg, i.p.). The neuroanatomical dissections were performed 45 min after the ethanol administration. The Western blot analyses were performed following the protocol described by Maas et al. (2005). Briefly, brains were homogenized in a buffer containing (1 % Nonidet P-40, 20 mM Tris-HCl (pH 8), 130 mM sodium chloride, 10 mM sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM DTT, 1 mM Na_3VO_4, and 1 mM PMSF), and then boiled for 5 min. The protein concentration was determined using Bradford protein assay. Proteins were separated by SDS-PAGE gels and transferred to polyvinylidene difluoride membranes using standard techniques. Membranes were blocked with 5 % BSA in Tri-buffered saline and Tween 20 (TBST), and then incubated overnight with anti-phosphorylated PKA substrate motif, 1:6,000 (Cell Signaling Technology, Beverly, MA). After washing with TBST, blots were incubated with HRP-conjugated secondary Ab anti-rabbit (1/20,000). Blots were developed using the ECL system (ECL Plus; Amersham Biosciences), and the intensities of the bands were quantified with SigmaGel image analysis software version 1.0 (Jandel Scientific, Madrid, Spain). All samples were run in duplicate, and band intensities were normalized to saline controls.

Statistical analyses

For all locomotor activity experiments, horizontal distance traveled in centimeters in 20 min served as the dependent variable. Intake experiments evaluated the ethanol (g/kg) and saccharine (ml) consumed in 2 h as a dependent variable. Blood ethanol levels were measured as mg/ml. For the Western blot experiments, the band intensity values were averaged to obtain a single value for each subject, and then divided by the GAPDH loading control band intensity. The average of these normalized values in the saline-treated group was arbitrarily considered to be 100 %, and then used for calculations involving all the different experimental manipulations. One- or two-way analysis of variance (ANOVA) and post hoc comparisons with the Newman–Keuls test were used when appropriate. Statistical analyses were performed using Statistica 6.1 (StatSoft, Tulsa, OK) software package.

Results

Experiments 1 and 2: effects of W7 on ethanol-induced locomotor activity

Figure 1a reflects the effect of W7 on ethanol-induced behavioral stimulation. A significant main effect of the W7 pretreatment dose ($F(4, 88)=6.8$; $p<0.01$) was revealed by a two-way ANOVA. Mean comparisons based on a significant W7 pretreatment × ethanol treatment interaction ($F(4, 88)=3.26$;

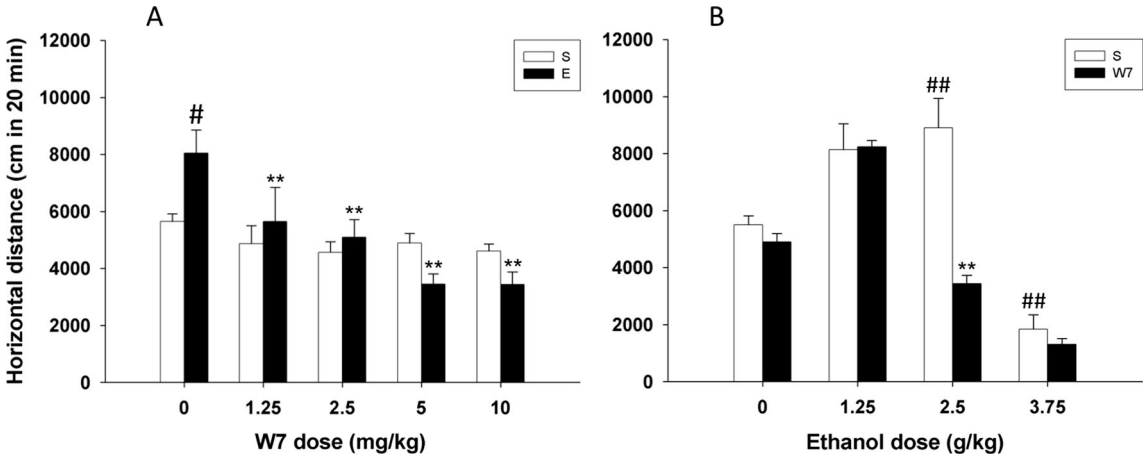

Fig. 1 Effect of different doses of W7 on ethanol-induced locomotor activity. **a** Animals (n=12–15) were acutely pretreated with W7 (0, 1.25, 2.5, 5, and 10 mg/kg, i.p.) 30 min before ethanol (0 or 2.5 g/kg, i.p.). Locomotor activity was recorded for 20 min immediately after ethanol challenge. Shown results are expressed as mean±SEM. $^{\#}p<0.01$, significantly different from saline–saline pretreated group; $^{**}p<0.01$, significantly different from saline–ethanol pretreated group. *S* saline, *E* ethanol. **b** Effect of different doses of ethanol in W7-pretreated animals (n=12–15). Mice were pretreated with W7 (0 and 5 mg/kg, i.p.) 30 min before ethanol injection (0, 1.25, 2.5, and 3.75 g/kg, i.p.), and locomotor activity was recorded for 20 min. Results are expressed as mean± SEM. $^{\#\#}p<0.01$, significantly different from saline–saline-treated group; $^{**}p<0.01$, significantly different from its respective saline-treated group. *S* saline, *W7* W7

$p<0.01$) revealed that W7 reduced the ethanol-induced stimulation. Newman–Keuls post hoc comparisons among ethanol-treated groups showed significant differences at the doses of 1.25, 2.5, 5, and 10 mg/kg ($p<0.01$) when compared with the saline–ethanol group. The W7 pretreatment did not affect locomotor responses after saline treatment. Figure 1b shows the effect of W7 (5 mg/kg, i.p.) on the locomotor effects induced by different doses of ethanol (0, 1.25, 2.5, and 3.75 g/kg, i.p.). Results of a two-way ANOVA showed a significant effect of ethanol dose ($F(3, 65)=52.76$; $p<0.01$) as well as the W7 pretreatment dose ($F(1, 65)=16.56$; $p<0.01$). A significant W7 pretreatment dose×ethanol treatment dose interaction was obtained ($F(3, 65)=11.92$; $p<0.01$). Post hoc Newman–Keuls comparisons among groups pretreated with saline showed significant differences between locomotion scores induced by 1.25, 2.5, or 3.75 g/kg ($p<0.01$) and those measured after saline treatment. Follow-up analyses also showed significant differences between saline- and W7-pretreated groups when ethanol was administered at a dose of 2.5 g/kg ($p<0.01$). No effects of this compound were found in animals treated with saline or ethanol at 1.25 or 3.75 g/kg.

Experiment 3: time course pattern of W7 effects on ethanol-induced stimulation

Figure 2 shows the time-course effect between W7 (5 mg/kg) pretreatment and ethanol (2.5 g/kg) treatment groups. Two-way ANOVA (pretreatment×time) demonstrated a significant main effect for the pretreatment ($F(1, 59)=11.33$; $p<0.01$) as well as the time factor ($F(2, 59)=4.39$; $p<0.05$). A significant interaction between both factors (pretreatment×time) was found ($F(2, 59)=8.66$; $p<0.05$). Newman–Keuls post hoc comparisons indicated that W7 was effective in reducing ethanol-stimulating effects at 15 and 30 min after pretreatment.

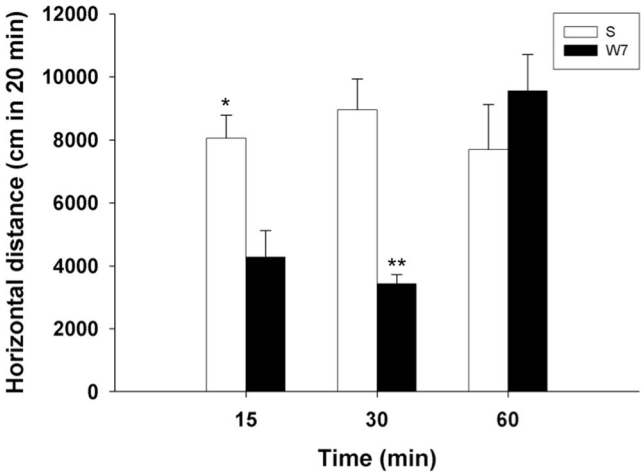

Fig. 2 Time course effect of pretreatment with W7 on ethanol-induced locomotor activity. Mice ($n=12$–15) were pretreated with W7 (0 or 5 mg/kg, i.p.) 15, 30, or 60 min before ethanol challenge (2.5 g/kg, i.p.), and locomotor activity was registered. Values represent mean distance traveled (cm)±SEM for all treatment groups. *$p<0.05$; **$p<0.01$, significantly different from its respective control group. *S* saline, *W7* W7

Experiment 4: effects of W7 on locomotor stimulation produced by other stimulant drugs

Figure 3 shows the effects of W7 (5 mg/kg) on cocaine- (4 mg/kg) and caffeine-induced (15 mg/kg) locomotor stimulations. Treatment with caffeine (A) ($F(1, 45)=43.4$; $p<0.01$) and cocaine (B) ($F(1, 40)=97.4$; $p<0.01$) produced significant increases in locomotor activity. No significant effect of W7 pretreatment was found either caffeine or cocaine.

Experiments 5 and 6: effect of several doses of W7 on voluntary ethanol and saccharine intake

Figure 4a shows the consequence of W7 administration (0, 2.5, 5, or 10 mg/kg, i.p.) on ethanol (10 %, *v*/*v*) consumption. Results from a one-way ANOVA indicated a significant effect of the W7 dose ($F(3, 44)=34.32$; $p<0.01$). Post hoc Newman–Keuls comparisons revealed that W7 administration reduced ethanol intake in a dose-dependent manner for all the doses of the study ($p<0.01$). As can be seen in Fig. 4b, W7 (5 or 10 mg/kg, i.p.) had no effect on voluntary saccharin intake. This conclusion was confirmed by a one-way ANOVA ($F(2, 45)=0.42$; $p>0.05$).

Experiment 7: effects of W7 on blood ethanol levels

As can be seen in Fig. 5, pretreatment with 5 mg/kg of W7 did not modify blood ethanol levels at the time of the behavioral testing. No significant differences were found (repeated measures two-way ANOVA; pretreatment×time) for the pretreatment (W7) or the interaction pretreatment×time.

Experiment 8: effect of W7 administration in vivo on ethanol-induced activated PKA footprint

Figure 6 shows the result of CaM inhibition with W7 on ethanol-induced PKA footprint in different brain areas. Results from a two-way ANOVA indicated a significant effect of the W7 pretreatment in the hypothalamus (B) and striatum (D) ($F(1, 24)=13.6$; $p<0.01$) and ($F(1, 18)=7.3$; $p<0.01$), respectively. Moreover, an ethanol treatment factor effect was found for the cortex (A) and hippocampus (C) ($F(1, 22)=5.6$, $p<0.05$) and ($F(1, 25)=18.6$; $p<0.01$), respectively. Although a lack of effect of W7 pretreatment or ethanol treatment was found in some structures of the study, a significant interaction was obtained between factors in all the neuroanatomical areas of the study ($F(1, 22)=4.7$; $p<0.05$) cortex, ($F(1, 24)=9.6$, $p<0.01$) hypothalamus, ($F(1, 25)=9.2$; $p<0.01$) hippocampus, and ($F(1, 18)=12.1$; $p<0.01$) striatum.

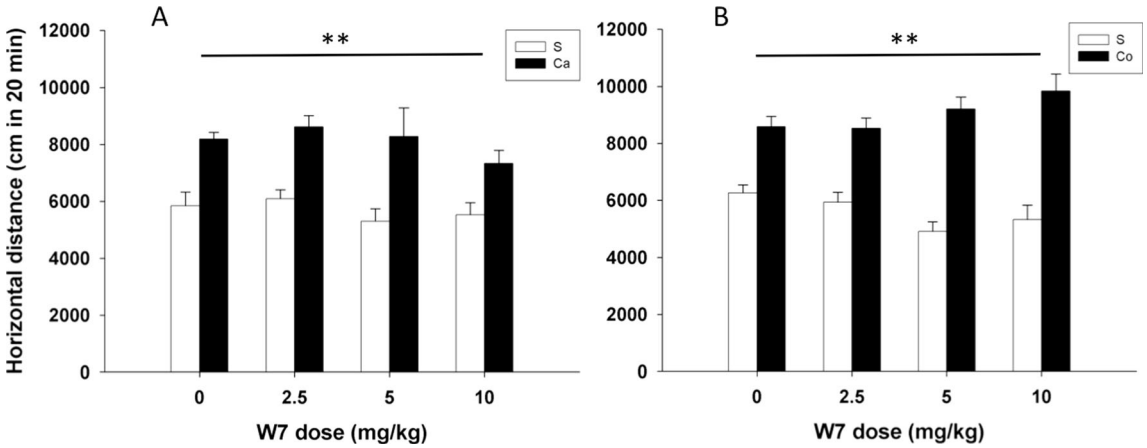

Fig. 3 Effects of W7 on the psychostimulant actions of caffeine (**a**) and cocaine (**b**). Animals (n=12) were pretreated with W7 (0, 2.5, 5, and 10 mg/kg, i.p.), and 30 min later, caffeine (15 mg/kg, i.p.) or cocaine (4 mg/kg, i.p.) was administered. Treatment with caffeine and cocaine produced significant increases in locomotor activity. Results expressed as mean±SEM. **p<0.01 significantly different from its respective saline group. *S* saline, *Ca* caffeine, *Co* cocaine

Post hoc Newman–Keuls comparisons among groups pretreated with saline showed significant differences after ethanol injection when compared with its saline control for all the structures of the study, **p<0.01 and p<0.05. Significant differences were found among saline–ethanol-treated groups and W7–ethanol-treated groups for all the structures of the study. $^{\#\#}p$<0.01 and $^{\#}p$<0.05. Furthermore, no significant differences were found among groups treated with W7 when compared with its respective control.

Discussion

The present study investigated the effects of CaM inhibition with W7 on ethanol-induced stimulation, ethanol intake, and ethanol-dependent PKA footprint. CaM blockade resulted in a reduction of ethanol-activating effects in a W7 dose-dependent manner. Doses of W7 that blocked the locomotor activation produced by ethanol did not modify the spontaneous locomotion of mice. Moreover, the suppression of the motor activity produced by high doses of this alcohol (Fig. 1b) was not affected by the pretreatment with W7. This would indicate that CaM blockade (at doses that reduced ethanol stimulation) had no effect on the motor-incoordinating, ataxic actions of ethanol (ethanol sedative effects). At this point, it is worth mentioning that in our conditions the W7 interaction with ethanol at a high dose is very near to the floor. Given this, an alternative interpretation of our results would involve a synergistic effect between ethanol at this dose (3.75 g/kg) and W7. We have not considered this possibility, as previous published results of our laboratory involving manipulations of the same proposed pathway did not increase ethanol sedative effects when

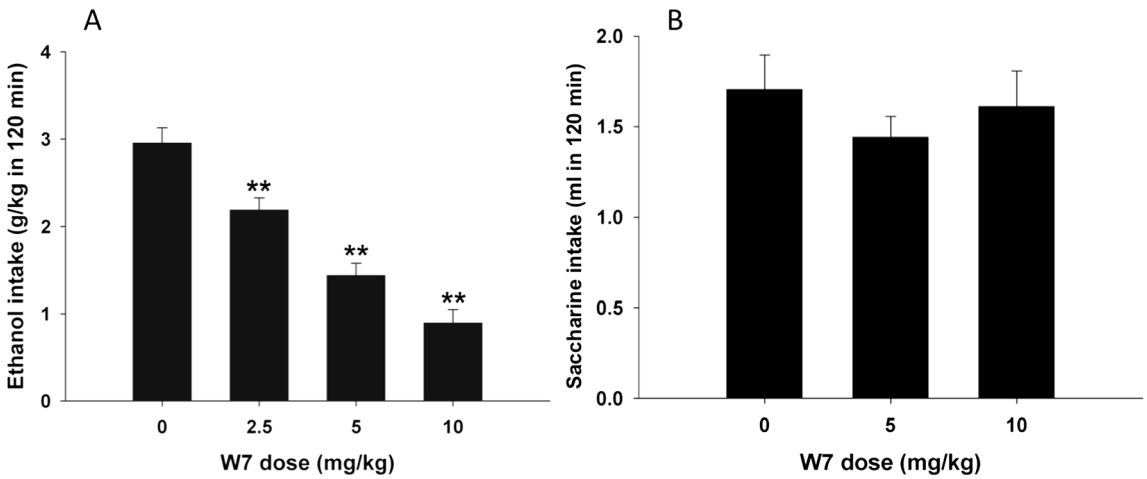

Fig. 4 Effect of different doses of W7 on ethanol drinking. Animals (n=16) were pretreated with W7 (0, 2.5, 5, and 10 mg/kg, i.p.) 30 min before ethanol (10 %, *v*/*v*) exposure. **a** Cumulative consumption of ethanol in 2 h. Results are expressed as mean±SEM of grams per kilogram consumed in 2 h. **p<0.01, significantly different from saline control group. **b** Effect of different doses of W7 on saccharine intake. Animals (n=16–18) were pretreated with W7 (0, 5, and 10 mg/kg, i.p.) 30 min before offering saccharine (0.1 %, *w*/*v*) solution. Results are expressed as mean±SEM of milliliters consumed in 2 h

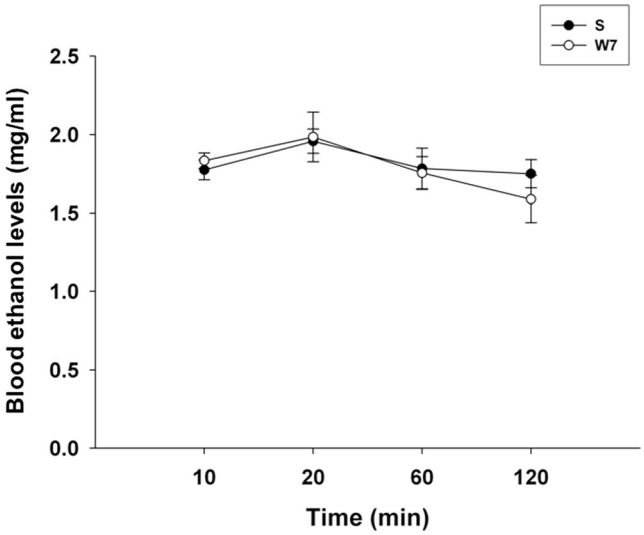

Fig. 5 Pharmacokinetic response to ethanol in Swiss CD-I mice. Animals (n=6) were injected with W7 (0 and 5 mg/kg, i.p.), and 30 min later, an ethanol (2.5 g/kg, i.p.) challenge was administered. A sample of 20 μl of blood was collected from the lateral tail vein at 10, 20, 60, and 120 min after ethanol injection. Immediately, blood ethanol levels were measured. Results are expressed as mean ± SEM

measured by a loss of righting reflex paradigm (Baliño et al. 2012; Tarragon et al. 2012). The W7 time-pattern response to ethanol demonstrated an interaction at short time intervals (15 and 30 min), whereas a recovering effect from the effects of W7 administration is observed at longer time intervals (60 min). It is of importance to remark at this point that CaM blockade seems to have a more prominent role on the behavioral-stimulating effect of ethanol when compared with that of other drugs with motor-stimulating properties. Doses of W7 that significantly reduced ethanol-induced stimulation did not alter locomotor activation produced by different psychostimulants such as caffeine or cocaine, at least at the doses and times tested in our experiments. Importantly, when measured during the time course of behavioral testing, this compound did not modify ethanol blood levels.

Conversely, regarding ethanol intake experiments, we have employed a daily limited-access model. In our experiments, control saline animals consumed amounts of ethanol above 2.5 g/kg. At this respect, it has been previously reported that these ethanol consumption produced a blood–ethanol concentration of approximately 1 mg/ml (≈25 mM), which is able to produce significant effects on physiology and behavior in mice (Rhodes et al. 2005; Tarragon et al. 2012). Our results also showed that pretreatment with the CaM inhibitor W7 dose-dependently reduced voluntary ethanol intake. In agreement with the W7 selectivity effects to ethanol in locomotion, it is of importance to remark that the suppressant effects of CaM manipulations on voluntary ethanol intake are probably not due either to an unspecific inhibition of general ingestive behavior or alterations in global taste sensation. Doses of W7 that reduced ethanol administration did not significantly reduce the consumption of sweetened water (Fig. 4b) or total fluid intake (data not shown).

As we have previously stated, the agent W7 acts as a high-affinity CaM blocker. In vitro, it has been reported that W7 can interact with other cellular substrates such as the troponin C and the myosin light-chain kinase (Frampton and Orchard 1992; Hoffman and Sykes 2009). In addition, W7 administration to cell cultures induced the inhibition of Na(+)–H+cellular exchange by the voltage-gated sodium channels (Burns et al. 1991). Despite these W7-unspecific effects, in our studies we have focused in W7-specific effects on cellular CaM.

The CaM has been described as a cellular Ca^{2+} sensor. The binding of Ca^{2+} alters the conformation of CaM, and the resulting CaM/Ca^{2+} complex can interact with target proteins and modulate their activity (Chin and Means 2000; Solà et al. 1999). Numerous CaM-binding proteins (CaMBP) have been described in relation to a wide range of cellular functions, from a structural role to the regulation of gene expression (Lu and Means 1993). CaM is widely distributed in the mammal brain (Seto-Ohshima et al. 1983), being of special relevance in sites involved in neurotransmission (Grab et al. 1980; Sobue et al. 1982; Wood et al. 1980). Different CaMBP proteins have been described as critical CaM effectors such the AC, Ca^{2+}/ATPase, cyclic nucleotide phosphodiesterase, and different protein kinases (Klee and Haiech 1980). However, the role of these CaMBP in mediating the neurobehavioral actions of ethanol has not been studied in detail yet. Interestingly, different authors have described the participation of Ca^{2+}-activated CaM cascade as a major cellular response to ethanol in cultured neural cells (Conti et al. 2009; Diamond and Gordon 1997). In addition, it has been shown that this cellular response to ethanol is mediated trough the direct stimulation of the AC, especially AC1 and AC8 (Conti et al. 2009; Maas et al. 2005). Given this, one possible explanation between W7 and the central effects of ethanol in mice would involve a Ca^{2+}/AC-dependent mechanism. At this point, it is worth mentioning that one key effector of CaM, the myosin light-chain kinase (MLCK) acts as a critical mediator of the main regulatory pathways that control the muscular excitation–contraction coupling. Thus, some of the behavioral results obtained in this study could be alternatively explained by an unspecific effect of MLCK inactivation due to a CaM blockade by W7. However, as we have previously mentioned, W7 effects are highly selective for the stimulating and motivational properties of ethanol.

Interestingly, in agreement with our suggestion, of a Ca^{2+}/AC-dependent mechanism, several groups have provided evidence that alterations in the cAMP signaling system modulate the response to ethanol in vivo. In addition, the Ca^{2+}/CaM as well as the AC subtypes 1 and 8 have been considered as a potential locus of interaction between the cAMP signaling and Ca^{2+} signal transduction pathways (Schaefer et al. 2000; Wand et al. 2001; Wong et al. 1999). Thus, it has been demonstrated that mice lacking of

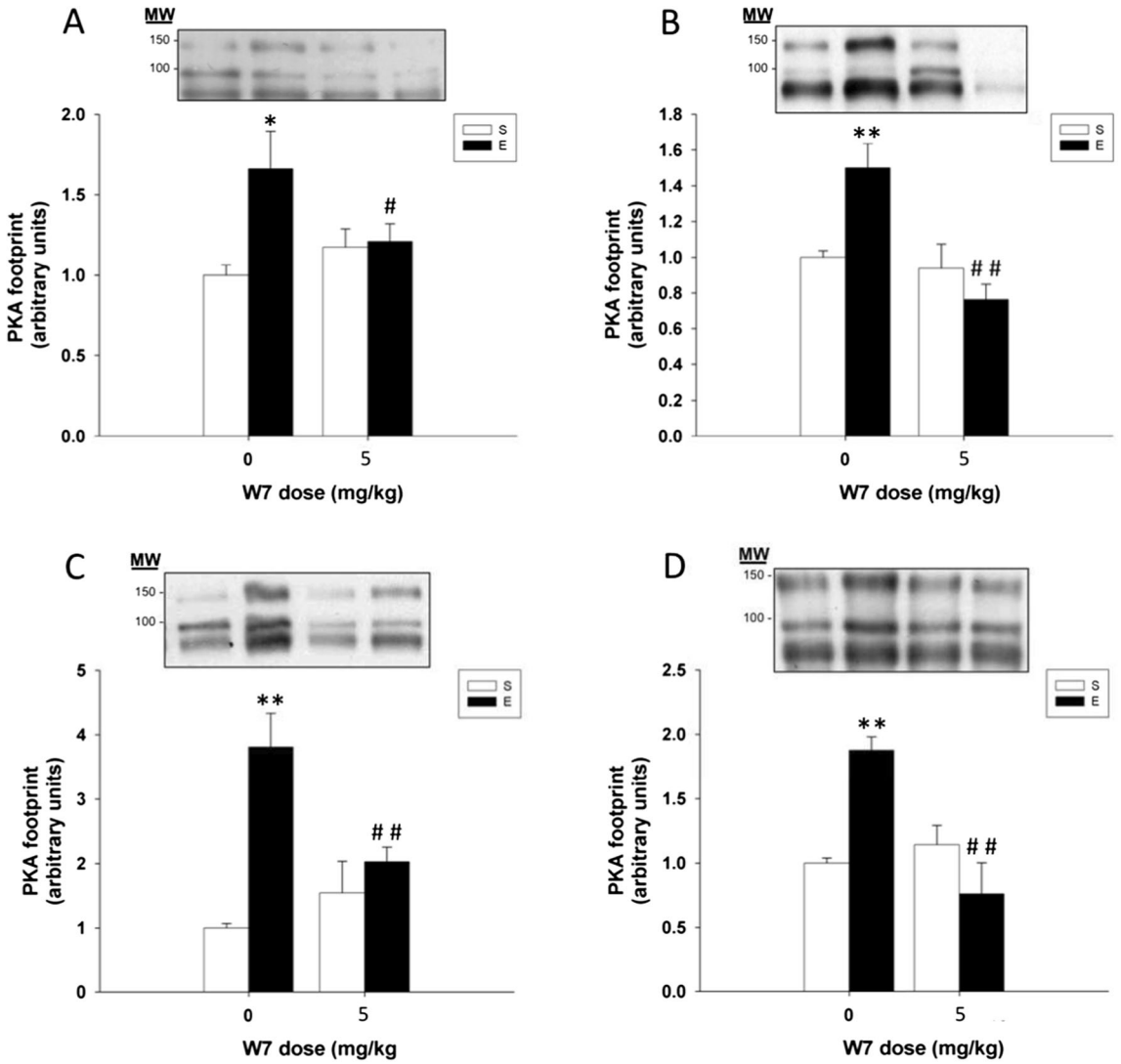

Fig. 6 Immunoblot analysis and quantification of the phosphorylated PKA substrate. Effects of W7 on the ethanol-induced PKA fingerprint. Animals were pretreated with W7 (0 and 5 mg/kg, i.p.) 30 min before ethanol (0 or 2.5 g/kg, i.p.) challenge. Brains were removed and dissected 45 min after an ethanol injection. Protein extracts (20 μg protein/lane) were analyzed on Western blot with an anti-phosphorylated PKA substrate motif antibody (1:6,000). *Top*, a representative Western blot of the PKA fingerprint after ethanol administration. Depicted is the densitometric analysis performed to quantify the relative intensity of the bands. **a** Cortex, **b** hypothalamus, **c** hippocampus, and **d** striatum. Depicted is the mean±SEM. $^{**}p<0.01$; $^{*}p<0.05$, significantly different from its respective saline control; $^{\#\#}p<0.01$; $^{\#}p<0.05$, significantly different from the saline–ethanol-treated group. *S* saline, *E* ethanol

AC1 and/or AC8 responded differently to ethanol administration when compared with wild-type animals. Interestingly, AC8 and AC1 knock-out mice showed a decreased ethanol-induced locomotor activity and ethanol intake and presented a variety of deficits in long-term depression and long-term potentiation (Villacres et al. 1998; Wang et al. 2003). In contrast to our results, previous published results demonstrate that mice lacking AC1 and AC8 presented an increased ethanol-induced sedation when compared with control mice (Maas et al. 2005). As can be observed in Fig. 1b in our experiments, locomotor depression produced by high ethanol dose (3.75 g/kg, i.p.) was not affected by the pretreatment with W7. At this respect, some strain-dependent differences in the response to ethanol and/or the neuronal adaptations carried out by the knock-out mice could explain these discrepancies. Therefore, convergent support indicates that there is a dose range of CaM inhibition, which appears to be able to block the stimulant effects of ethanol in mice without increasing the depressant/ataxic effects of this alcohol.

Consistent with our ethanol intake experiments, convergent support indicates that manipulations of different Ca^{2+}-dependent CaM cascade intermediates were effective in reducing the ingestive ethanol behavior in mice (Baliño et al. 2012; Maas et al. 2005; Tarragon et al. 2012). In all cases, tap or sweetened water drinking was unaffected.

As stated previously, the activated Ca^{2+}/CaM leads to an increase of AC activity. These activated AC convert ATP to cAMP and have been suggested as critical mediators of the cAMP/PKA cascade in response to ethanol administration (Conti et al. 2009). Thus, in this work we have investigated the cellular functional consequences of such ethanol-induced kinase activation. Similarly to Maas et al.'s (2005), we have used a subset of phosphorylable substrate consensus motifs as a PKA-activated fingerprint. These authors demonstrated that mice treated with ethanol showed an increased intensity of several bands that represented different phosphorylated PKA-dependent consensus motif. These bands that were increased after ethanol treatment in the WT brain were compromised in the AC1 and AC8 knock-out mice brain. In agreement, our results showed an increased intensity of the same molecular weight Western blot bands (Fig. 5) after ethanol administration in the brain areas of the study. We have focused in four different brain regional anatomical areas that have been proved to be involved in this ethanol-induced activation of PKA. In accordance, immunohistochemical studies have demonstrated an increased activity of the cAMP/PKA signaling cascade in different brain areas that parallel our Western blot experiments, such as the prefrontal cortex, the medial and lateral septum, the basolateral amygdala, the paraventricular and anterior hypothalamus, hippocampus, dentate gyrus, nucleus accumbens, and the ventral tegmental area (Asyyed et al. 2006; Ostroveanu et al. 2007). Moreover, these neuroanatomical areas have been proposed as specialized reward areas critical for the behavioral actions elicited by ethanol. Conversely, when W7 was administered prior to ethanol, the band densitometry analyses indicate decreased band intensity when compared with its respective controls as a result of a decreased Ca^{2+}/CaM-dependent PKA activation by ethanol. This would indicate that CaM activity is critical to further activate such cAMP/PKA cascade in response to ethanol.

Ethanol effects on Ca^{2+} flux and distribution have been extensively studied in different neuronal cell preparations (Belia et al. 1995; Davidson et al. 1988; Chan and Greenberg, 1991). In addition, acute ethanol administration modulates Ca^{2+} flux in vitro, and it is generally accepted that ethanol concentrations (<80 mM) increase intracellular Ca^{2+} levels (Daniell and Harris 1989; Mironov and Hermann 1996; Xiao et al. 2005). It has been described that the cytosolic Ca^{2+} increase can be achieved as the result either of the Ca^{2+} extracellular influx or its release from internal stores. Moreover, the cytosolic Ca^{2+} concentration is controlled by active Ca^{2+} sequestration into intracellular stores (endoplasmic reticulum and mitochondria) and by Ca^{2+} binding to intracellular proteins (Belia et al. 1995; González et al. 2007; Kelm et al. 2007; Leslie et al. 1990; Salazar et al. 2008).

At this respect, we have previously demonstrated that extracellular Ca^{2+} flux as well as intracellular Ca^{2+} levels appear to determine the neurobehavioral response to ethanol. In addition, our group has demonstrated that intracellular Ca^{2+} homeostasis is critical to the stimulatory effects of ethanol and ethanol intake in vivo. Animals pretreated with the ER ryanodine receptor inhibitor dantrolene, presented a decreased ethanol-induced locomotor activity when systemically administered and also a decrease in the ethanol ingestive behavior using a limited-access paradigm (Tarragon et al. 2012). Moreover, intracellular Ca^{2+} chelation with BAPTA-AM was able to modulate selectively the activating effects of ethanol as well as the ethanol intake (Baliño et al. 2012). In other words, manipulations affecting Ca^{2+} flux through voltage-dependent calcium channels were also effective in reducing the stimulating effects of ethanol in a dose-dependent manner when systemically administered (Baliño et al. 2010). Thus, the behavioral response to acute ethanol administration appears to share the same molecular mechanism independently as Ca^{2+} enters from the extracellular side or its release from the intracellular pools.

Interestingly, recent published data from our laboratory (Baliño et al. 2013) have demonstrated that there is a different molecular mechanism implicated in the acute response to ethanol depending on the source of Ca^{2+}. These experiments have demonstrated that acute ethanol administration in vivo promotes an increased activity of the cAMP/PKA cascade. Manipulations affecting Ca^{2+} release from the ER appeared to be critical to further activate the cAMP/PKA cascade, as animals pretreated with dantrolene did not show an activated PKA response after ethanol administration. On the contrary, manipulations of Ca^{2+} flux through voltage-dependent calcium channels did not modify the activation of PKA after ethanol administration. Thus, the pretreatment with the calcium channel blocker diltiazem did not interfere with the ethanol-induced activation of PKA in mice.

Taking together all these results, we have hypothesized a linear pattern of Ca^{2+}-dependent events elicited by ethanol, which primarily involves Ca^{2+} release through ryanodine receptor to further activate CaM and the cAMP/PKA signaling pathway, leading to voltage-dependent calcium channels modulation. This would explain how a different cellular Ca^{2+} manipulations share the same functional consequences in a rodent model. In agreement with our suggestion that involves a linear sequence of events, it has been shown that Ca^{2+}/CaM facilitates L-type channel activation through a cAMP/PKA-dependent mechanism (Marcantoni et al. 2009; Zühlke et al. 1999).

In summary, these data demonstrate a critical role of CaM in mediating the behavioral response elicited by acute ethanol administration in mice. Thus, the CaM blockade with W7 was effective in reducing the activating effects produced by ethanol. Furthermore, the pretreatment with W7 blocked selectively the ethanol ingestive behavior in mice studied with a limited-access intake paradigm. The PKA activation by ethanol is also prevented in animals pretreated with W7. All these results with previous literature demonstrate a pivotal role of

Ca^{2+}-dependent events elicited by ethanol that support the hypothesis of a cAMP/PKA-dependent activation pathway as a possible mechanism of action of ethanol in the CNS. Current studies of our laboratory are focused on elucidating the particular role of such Ca^{2+}-dependent pathways in mediating the behavioral response to ethanol.

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