Acta Pharmacologica Sinica (2009) 30: 1421–1427

www.nature.com/aps

Original Article

The impact of extracellular and intracellular Ca^{2+} on ethanol-induced smooth muscle contraction

Naciye YAKTUBAY DÖNDAŞ*, Mahir KAPLAN, Derya KAYA, Ergin ŞİNGİRİK

Department of Pharmacology, Faculty of Medicine, Çukurova University, 01330 Adana, Turkey

Aim: To evaluate the impact of extracellular and intracellular Ca^{2+} on contractions induced by ethanol in smooth muscle.
Methods: Longitudinal smooth muscle strips were prepared from the gastric fundi of mice. The contractions of smooth muscle strips were recorded with an isometric force displacement transducer.
Results: Ethanol (164 mmol/L) produced reproducible contractions in isolated gastric fundal strips of mice. Although lidocaine (50 and 100 μmol/L), a local anesthetic agent, and hexamethonium (100 and 500 μmol/L), a ganglionic blocking agent, failed to affect these contractions, verapamil (1–50 μmol/L) and nifedipine (1–50 μmol/L), selective blockers of L-type Ca^{2+} channels, significantly inhibited the contractile responses of ethanol. Using a Ca^{2+}-free medium nearly eliminated these contractions in the same tissue. Ryanodine (1–50 μmol/L) and ruthenium red (10–100 μmol/L), selective blockers of intracellular Ca^{2+} channels/ryanodine receptors; cyclopiazonic acid (CPA; 1–10 μmol/L), a selective inhibitor of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase; and caffeine (0.5–5 mmol/L), a depleting agent of intracellular Ca^{2+} stores, significantly inhibited the contractile responses induced by ethanol. In addition, the combination of caffeine (5 mmol/L) plus CPA (10 μmol/L), and ryanodine (10 μmol/L) plus CPA (10 μmol/L), caused further inhibition of contractions in response to ethanol. This inhibition was significantly different from those associated with caffeine, ryanodine or CPA. Furthermore the combination of caffeine (5 mmol/L), ryanodine (10 μmol/L) and CPA(10 μmol/L) eliminated the contractions induced by ethanol in isolated gastric fundal strips of mice.
Conclusion: Both extracellular and intracellular Ca^{2+} may have important roles in regulating contractions induced by ethanol in the mouse gastric fundus.

Keywords: ethanol; contraction; smooth muscle; verapamil; nifedipine; ryanodine receptors; ryanodine; ruthenium red; caffeine

Acta Pharmacologica Sinica (2009) 30: 1421–1427; doi: 10.1038/aps.2009.123; published online 14 Sep 2009

Introduction

Alcohol (ethanol) in its various forms is used for both medicinal and social purposes. The widespread use of ethanol has led to considerable efforts to define the mechanisms of its action. Recently, it has been suggested that ethanol interferes with excitation-contraction mechanism[1]. Some studies have evaluated the possible role of Ca^{2+} to clarify this phenomenon[1–3]. However, the underlying mechanisms of this effect are still not fully understood. Additionally, existing experimental results are controversial. For example, Belia *et al* posited that the increment of Ca^{2+} by ethanol is considered to be the consequence of activation of L-type voltage-dependent calcium channels[1]. In contrast Oz *et al* suggest that ethanol inhibits the function of voltage-dependent Ca^{2+} channels[4]. Similarly, controversial results have been reported relating to the effect of ethanol on intracellular Ca^{2+} levels. For example, Werber *et al* reported that ethanol could evoke Ca^{2+} release from intracellular stores in arterial smooth muscle cells[2]. In contrast, Cofan *et al* suggest that ethanol can decrease intracellular calcium ion transients in skeletal muscle[3]. Therefore, in the present study, we aimed to clarify the relationship between Ca^{2+} and the excitation-contraction mechanisms of gastric smooth muscle by ethanol. Ca^{2+} plays a major role in the regulation of cell functions. This ion makes its entrance into the cytoplasm either from outside the cell through the cell membrane via calcium channels, or from internal calcium storages. Therefore, in the present study, to evaluate the role of Ca^{2+} we examined the role of both extracellular and intracellular Ca^{2+} on contractions induced by ethanol in the gastric fundi of mice.

* To whom correspondence should be addressed.
E-mail yakdas25@cu.edu.tr
Received 2009-05-06 Accepted 2009-07-10

Materials and methods

Animals and experimental design

Swiss albino mice of either sex, weighing 20–25 g, were used for the experiments. Approximately equal numbers of each sex were used in each experimental group. The experimental

procedures were approved by the animal care committee of the University of Çukurova (TIBDAM), and the experiments were carried out in accordance with the Principles of Laboratory Animal Care (National Institutes of Health guideline; publication No 86-23, reversed 1984). All animals were kept under standard laboratory conditions (12 h dark/12 h light).

Tissue preparation

Mice were fasted for 24 h with free access to water, then killed by stunning and cervical dislocation. The stomach was removed and longitudinal muscle strips (approximately 15 mm×3 mm) were prepared from the gastric fundus (one strip from each animal). The strips were then mounted under a resting tension of 0.5 g in 10 mL organ baths containing Tyrode's solution (mmol/L: NaCl 136.7, KCl 2.6, $CaCl_2$ 1.8, $MgCl_2{\cdot}6H_2O$ 0.95 $NaH_2PO_4{\cdot}2H_2O$ 0.41, $NaHCO_3$ 11.9, glucose 5.05). The bath medium was maintained at 37 ºC and bubbled with 95% O_2 and 5% CO_2. Each preparation was washed with fresh Tyrode's solution at 15 min intervals during a 1 h equilibration period. The responses were recorded with an isometric force displacement transducer (MAY, FDT 0.5). Data were recorded and stored using data acquisition software (BIOPAC, MP35 System, Inc).

Protocol

In the present study, two sets of experiments were performed, each of which is detailed below.

In the first set of experiments, after a preincubation period of 1 h, the basal tonus of the preparation was recorded for 5 min and then ethanol (164 mmol/L) was added to the organ baths. The addition of ethanol resulted in contractions reaching a steady state within 10 min. The tissue was then rinsed with Tyrode's solution and allowed to rest for 40 min. After resting, the protocol was repeated. This set of experiments served as the general control group (n=12).

The second set of experiments was conducted after the first ethanol (164 mmol/L) response was recorded and the tissue was rinsed with Tyrode's solution. Preparations were incubated separately with lidocaine (50–100 μmol/L), hexamethonium (100–500 μmol/L), verapamil (1, 10, 50 μmol/L), nifedipine (1, 10, 50 μmol/L), Ca^{2+}-free medium (Ca^{2+}-free Tyrode's solution; mmol/L: NaCl 139.4, KCl 2.6, $MgCl_2{\cdot}6H_2O$ 0.95 $NaH_2PO_4{\cdot}2H_2O$ 0.41, $NaHCO_3$ 11.9, glucose 5.05), ryanodine (1, 10, 50 μmol/L), ruthenium red (10, 50, 100 μmol/L), cyclopiazonic acid (CPA; 1, 5, 10 μmol/L), caffeine (0.5, 1, 5 mmol/L), caffeine (5 mmol/L)+CPA (10 μmol/L), ryanodine (10 μmol/L)+CPA (10 μmol/L), or caffeine (5 mmol/L)+ryanodine (10 μmol/L)+CPA (10 μmol/L). After a 40-min incubation, the second ethanol (164 mmol/L) response was examined in the presence of the drug(s) used in the incubation period. Each concentration of each substance was administered to a separate group (n=5–6). After the drug was washed away, a third application of ethanol was carried out. The results of this application were not included in the statistical analysis.

Chemicals

Ethanol unhydrous, hexamethonium, lidocaine, verapamil, nifedipine, ryanodine, ruthenium red, cyclopiazonic acid, and caffeine were obtained from Sigma Chemical Co, St Louis, MO, USA. Stock solutions of hexamethonium, lidocaine, verapamil, ryanodine, ruthenium red, and caffeine were dissolved in distilled water. Nifedipine and cyclopiasonic acid were dissolved in dimethylsulfoxide (DMSO; 1/20000 dilution).

Statistical analysis

In each experiment in this study, the first two contractile responses to ethanol (164 mmol/L) were used in statistical analysis. Firstly, a response was obtained in the absence of the drug; this was used as the control response for the strip and was regarded as equal to 100%. The second response was obtained in the presence of the drug (in the general control group, the first and the second contractile responses of ethanol were obtained in the absence of the drug). For each preparation, the second contractile response to ethanol was calculated as a percentage of the first response in the same tissue. The results of this calculation were then compared with the results obtained from the general control group. All data, except for the experimental results of using the Ca^{2+}-free medium, were analyzed statistically by one-way analyses of variance (ANOVA) followed by Bonferroni corrections. Experimental results from the Ca^{2+}-free medium were analyzed statistically with the use of unpaired Student's t tests. A value of P<0.05 was considered statistically significant.

Results

Control experiments

Ethanol produced reproducible contractions in the isolated gastric fundal strips of mice. In each experiment, two contractile responses were analyzed (Table 1, Figure 1). In all experiments, after the experimental protocol was completed, the preparation was washed and the third contractile response was obtained.

Table 1. The contractile effect of ethanol (164 mmol/L) in isolated gastric fundal strips of mice. For each group, the points are means±SEM of gastric fundal strips from 12 animals.

Group	First application of 164 mmol/L EtOH (mN)	Second application of 164 mmol/L EtOH (mN)
Control	1023.1±126.4	1251.4±98.9
DMSO Control (1/20000 dilution)	1003.5±64.6	1209.3±69.5

Effects of lidocaine and hexamethonium

Lidocaine (50 and 100 μmol/L), a local anesthetic agent, and hexamethonium (100–500 μmol/L), a ganglionic blocking agent, failed to affect the contractions induced by ethanol in the isolated gastric fundal strips of mice (Table 2). These

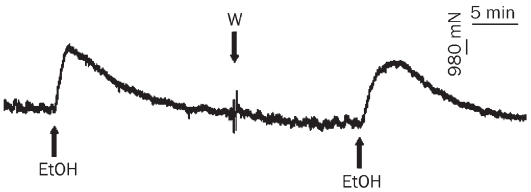

Figure 1. A typical tracing showing reproducible contractile responses induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice (general control group). EtOH: ethanol, W: wash, mN: milli Newton.

Table 2. Effects of lidocaine (50–100 μmol/L) and hexamethonium (100–500 μmol/L) on contractions induced by ethanol (EtOH; 164 mmol/L) in isolated gastric fundal strips of mice. For each concentration of the drug, the points are means±SEM of gastric fundal strips from 6 animals.

Drug Name	Drug Concentration (μmol/L)	EtOH (% Contraction)	EtOH+Drug (% Contraction)
Lidocaine	50	104.4±12.9	128.1±11.5
	100	104.4±12.9	112,9±10.6
Hexamethonium	100	104.4±12.9	113.2±13.3
	500	104.4±12.9	120.4±10.1

agents did not affect the basal tones of the preparations (wash-out response of lidocaine group: 121.6±12.0; wash-out response of hexamethonium group: 123.4±13.6).

Effect of extracellular Ca^{2+} on ethanol-induced contractions

Verapamil (1, 10, and 50 μmol/L), a selective blocker of L-type Ca^{2+} channels, significantly inhibited (P<0.05) the contractile responses to ethanol (Figures 2A, 2B) at all of the concentrations used in this study. Moreover, this agent slightly decreased the basal tones of the preparations (Figure 2A; wash-out response of this group: 120.5±10.2). Similarly, nifedipine (1, 10, 50 μmol/L), a selective blocker of L-type Ca^{2+} channels, significantly inhibited (P<0.05) contractile responses to ethanol (Figures 3A, 3B) at all concentrations used in this study. Like verapamil, nifedipine also slightly decreased the basal tones of isolated gastric fundal strips of mice (Figure 3A; wash-out response: 91.3±5.8). The Ca^{2+}-free medium nearly eliminated contractile responses to ethanol (Figure 4A, 4B), and caused a dramatic inhibition of basal tone (Figure 4A; wash out response: 128.6±10.0).

Effects of intracellular Ca^{2+} on ethanol-induced contractions

The highest concentration (50 μmol/L) of ryanodine, a selective blocker of intracellular Ca^{2+} channels/ryanodine receptors, significantly inhibited (P<0.05) the contractile responses to ethanol (Figure 5A, 5B). The lower concentrations (1, 10 μmol/L) of this drug decreased the effect of ethanol (Figure 5B). This agent did not cause any significant alterations in the basal tones of the preparations (Figure 5A; wash-out response

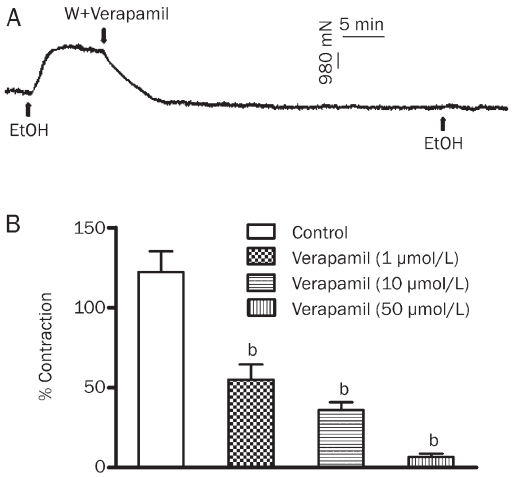

Figure 2. (A) A representative trace showing the effect of verapamil (50 μmol/L) on contractile responses to ethanol (164 mmol/L). EtOH: Ethanol, W: Wash, mN: milli Newton. (B) Effect of verapamil (1, 10, 50 μmol/L) on contractions induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice. Control: 164 mmol/L ethanol. For each concentration of the drug, the points are means±SEM of gastric fundal strips from 6 animals. Significant differences were evaluated by one-way analysis of variance (ANOVA). *Post hoc*: Bonferroni. ^{b}P<0.05 *vs* control.

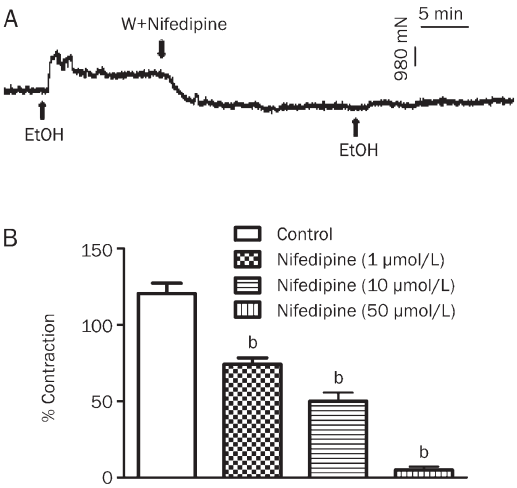

Figure 3. (A) A representative trace showing the effect of nifedipine (50 μmol/L) on contractile responses to ethanol (164 mmol/L). EtOH: Ethanol, W: Wash, mN: milli Newton. (B) Effect of nifedipine (1, 10, 50 μmol/L) on contractions induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice. Control: 164 mmol/L ethanol includes DMSO (1:20000 dilution). For each concentration of the drug, the points are means±SEM of gastric fundal strips from 6 animals. Significant differences were evaluated by one-way analysis of variance (ANOVA). *Post hoc*: Bonferroni. ^{b}P<0.05 *vs* control.

of this group: 25±3.1). The effect of this agent was irreversible. The other selective blocker of ryanodine receptors, ruthenium red (10, 50, 100 μmol/L), significantly inhibited (P<0.05) the contractile responses to ethanol at all concentrations used

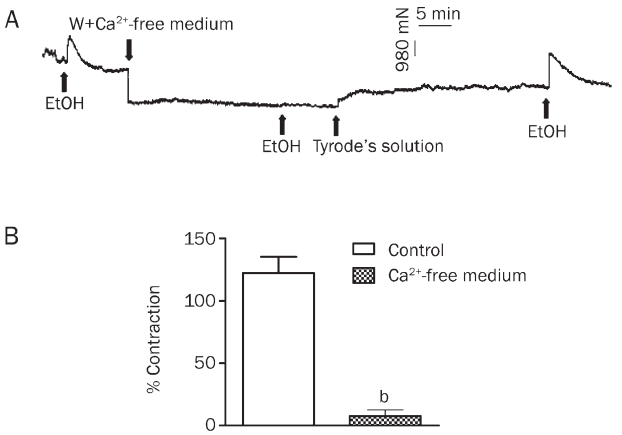

Figure 4. (A) A representative trace showing the effect of Ca^{2+}-free medium on contractile responses to ethanol (164 mmol/L). EtOH: Ethanol, W: Wash, mN: milli Newton. (B) Effect of Ca^{2+}-free medium on contractions induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice. The points are means±SEM of gastric fundal strips from 6 animals. Significant differences were evaluated by unpaired Student's *t* tests. $^{b}P<0.05$ *vs* control.

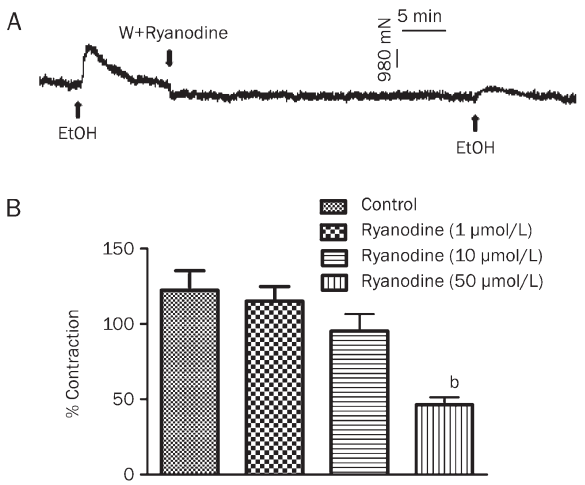

Figure 5. (A) A representative trace showing the effect of ryanodine (50 μmol/L) on contractile responses to ethanol (164 mmol/L). EtOH: Ethanol, W: Wash mN: milli Newton. (B) Effect of ryanodine (1, 10, 50 μmol/L) on contractions induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice. Control: 164 mmol/L ethanol. For each concentration of the drug, the points are means±SEM of gastric fundal strips from 5 animals. Significant differences were evaluated by one-way analysis of variance (ANOVA). *Post hoc*: Bonferroni. $^{b}P<0.05$ *vs* control.

(Figure 6A, 6B). Like ryanodine, it did not cause any significant alterations in the basal tones of the preparations (Figure 6A; wash-out response of this group: 118.4±9.8). Cyclopiazonic acid (CPA; 1, 5, 10 μmol/L), a selective inhibitor of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, significantly inhibited ($P<0.05$) the ethanol-induced contractions at all concentrations used in this study (Figure 7A, 7B). At the beginning of the experiments, CPA only slightly and transiently increased

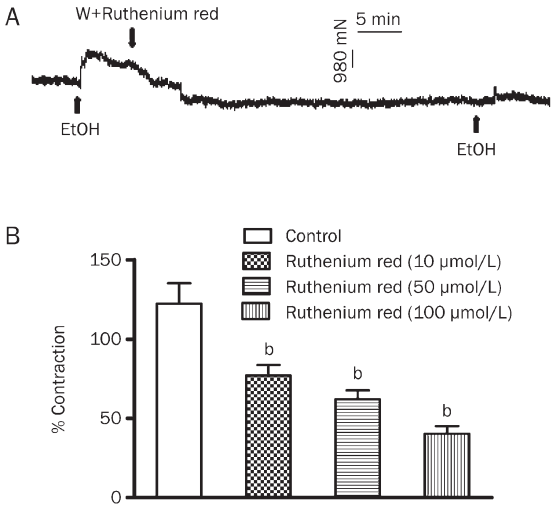

Figure 6. (A) A representative trace showing the effect of ruthenium red (100 μmol/L) on contractile responses to ethanol (164 mmol/L). EtOH: Ethanol, W: Wash, mN: milli Newton. (B) Effect of ruthenium red (10, 50, 100 μmol/L) on contractions induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice. Control: 164 mmol/L ethanol. For each concentration of the drug, the points are means±SEM of gastric fundal strips from 6 animals. Significant differences were evaluated by one-way analysis of variance (ANOVA). *Post hoc*: Bonferroni. $^{b}P<0.05$ *vs* control.

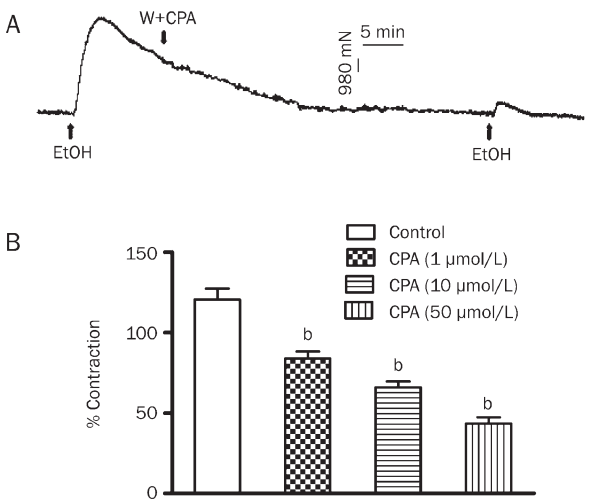

Figure 7. (A) A representative trace showing the effect of cyclopiazonic acid (CPA; 10 μmol/L) on contractile responses to ethanol (164 mmol/L). EtOH: Ethanol, W: Wash, mN: milli Newton. (B) Effect of CPA (1, 5, 10 μmol/L) on contractions induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice. Control: 164 mmol/L ethanol includes DMSO (1:20000 dilution). For each concentration of the drug, the points are means±SEM of gastric fundal strips from 5 animals. Significant differences were evaluated by one-way analysis of variance (ANOVA). *Post hoc*: Bonferroni. $^{b}P<0.05$ *vs* control.

the basal tone of the preparation (Figure 7A). This increment lasted about 5 min, after which the effects decreased. Between 40 and 50 min of the incubation time, which corresponds

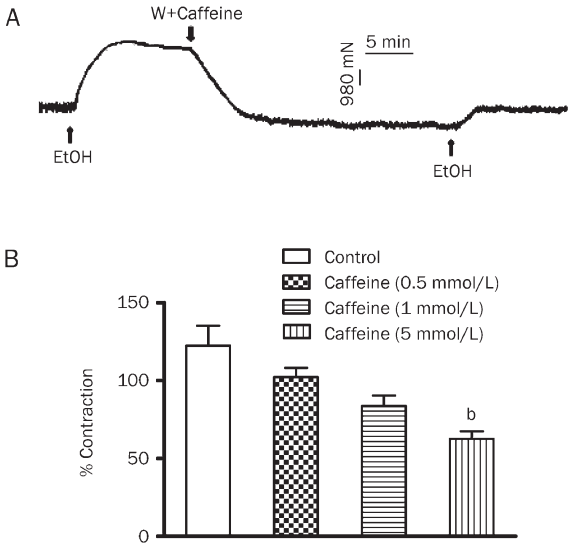

Figure 8. (A) A representative trace showing the effect of caffeine (5 mmol/L) on contractile responses to ethanol (164 mmol/L). EtOH: Ethanol, W: Wash, mN: milli Newton. (B) Effect of caffeine CPA (0.5, 1, 5 mmol/L) on contractions induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice. Control: 164 mmol/L ethanol. For each concentration of the drug, the points are means±SEM of gastric fundal strips from 6 animals. Significant differences were evaluated by one-way analysis of variance (ANOVA). *Post hoc*: Bonferroni. $^{b}P<0.05$ *vs* control.

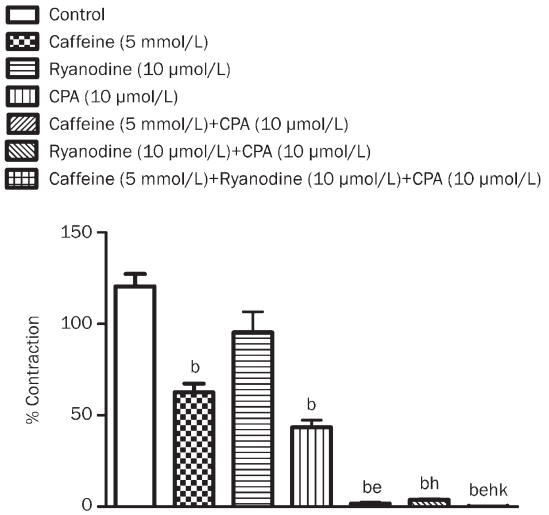

Figure 9. Effects of caffeine (5 mmol/L), ryanodine (10 μmol/L), cyclopiazonic acid (CPA; 10 μmol/L), caffeine (5 mmol/L)+CPA (10 μmol/L), ryanodine (10 μmol/L)+CPA (10 μmol/L) and caffeine (5 mmol/L)+ryanodine (10 μmol/L)+CPA (10 μmol/L) on contractions induced by ethanol (164 mmol/L) in isolated gastric fundal strips of mice. Control: 164 mmol/L ethanol includes DMSO (1:20000 dilution). For each concentration of the drug, the points are means±SEM of gastric fundal strips from 5–6 animals. Significant differences were evaluated by one-way analyses of variance (ANOVA). Post hoc: Bonferroni. $^{b}P<0.05$ *vs* control. $^{e}P<0.05$ *vs* caffeine (5 mmol/L). $^{h}P<0.05$ *vs* ryanodine (10 μmol/L). $^{k}P<0.05$ *vs* CPA (10 μmol/L).

to the time of the second ethanol application in the normal experimental protocol, CPA slightly increased the basal tone (wash-out response of this group: 110.5±13.4). Similar to ryanodine, caffeine, a depleting agent of intracellular Ca^{2+} stores, decreased the effect of ethanol in all concentrations used in this study (0.5, 1, 5 mmol/L). Only the highest concentration of caffeine caused a significant inhibition of contractile responses to ethanol (Figure 8B). This agent did not cause any significant alteration in the basal tones of the preparations (Figure 8A; wash-out response of this group: 104.1±9.7). The combination of caffeine (5 mmol/L)+CPA (10 μmol/L) potentiated the inhibitory action of caffeine (5 mmol/L) and CPA (10 μmol/L) on ethanol-induced contractions. This inhibition was significantly different ($P<0.05$) from both caffeine (5 mmol/L) and CPA (10 μmol/L) results (Figure 9). In addition, the combination of ryanodine (10 μmol/L)+CPA (10 μmol/L) also potentiated the inhibitory action of ryanodine (10 μmol/L) and CPA (10 μmol/L) on ethanol-induced contractions. This inhibition was significantly different ($P<0.05$) from those of both ryanodine (10 μmol/L) and CPA (10 μmol/L) (Figure 9). Furthermore, the combination of caffeine (5 mmol/L)+ryanodine (10 μmol/L)+CPA (10 μmol/L) completely eliminated the contractile responses to ethanol in the gastric fundal strips of mice (Figure 9).

Discussion

In our study, we aimed to investigate the role of Ca^{2+} on contractions induced by ethanol in the mouse gastric fundus. Since we obtained more stable contractile responses with 164 mmol/L (1 v/v) of ethanol in our preliminary experiments, we used this concentration in the current study. The experimental results of a study by Cooke and Birchall support our choice of this ethanol concentration[5]. In their study, the authors reported that gastric muscle is exposed to ethanol concentrations higher than those in blood. In addition, Sim *et al* also reported that a 342 mmol/L (2 v/v %) concentration of ethanol used in isolated cat gastric fundi appears to be attainable in the stomach muscle tissues of living animals[6]. In light of this, we believe that the ethanol concentration used in the present study is acceptable for use with the gastric fundus.

To clarify if the effects of ethanol are due to muscular or neuronal causes, we used hexamethonium and lidocaine to prevent neuronal action potentials. Hexamethonium is a ganglionic blocking agent[7]. It can act on receptors at pre-ganglionic sites in both the sympathetic and parasympathetic nervous systems, both of which are regulated only pre-ganglionically by nicotinic acetylcholine receptors[7]. Hexamethonium has no effect on the muscarinic acetylcholine receptors located on target organs of the parasympathetic nervous system, nor on nicotinic acetylcholine receptors at the neuromuscular junctions that are responsible for skeletal muscle motor response. Lidocaine, a local anesthetic agent, alters depolarization in neurons by blocking the fast voltage gated sodium (Na^{+}) channels in the cell membrane[8]. With sufficient blockage, the membrane of the presynaptic neuron will not depolarize and so will fail to transmit an action potential. With lidocaine, there is a high

degree of selectivity in the blockage of neurons and neuron signaling[8]. In this study, hexamethonium and lidocaine failed to affect the contractions induced by 164 mmol/L ethanol in isolated gastric fundal strips of mice. Our present data suggest that ethanol may directly affect the muscle cells of the gastric fundus. The results of a study conducted by Keshavarzian *et al* confirm our hypothesis[9]. Since the effects of ethanol in cat esophagi were not eliminated by cervical vagotomy or intravenous tetrodotoxin (TTX), the authors suggested that ethanol has a direct effect on the esophageal muscle cells of cats[9]. Similarly, Sim *et al* reported that the effect of ethanol was not inhibited by atropine (1 μmol/L), hexamethonium 1 μmol/L, phentolamine (1 μmol/L), propranolol (1 μmol/L) or TTX (1 μmol/L) in cat gastric smooth muscle[10]. Zhang *et al* also confirmed our hypothesis[11], and suggested that ethanol-induced contractions in canine cerebral vascular tissues are a result of direct actions on the smooth muscle cells[11].

Ca^{2+} plays a major role in the regulation of various cell functions. This ion enters the cytoplasm either from outside the cell through the cell membrane via calcium channels, such as voltage-gated calcium channels, or from internal calcium storages in sarcoplasmic/endoplasmic reticulum. Therefore, in the present study, to evaluate the role of Ca^{2+}, we examined the influence of both extracellular and intracellular Ca^{2+} on contractions induced by ethanol in the gastric fundal strips of mice.

In order to evaluate the role of extracellular Ca^{2+} on ethanol-induced contractions, we examined verapamil, nifedipine and a Ca^{2+}-free medium in separate experimental groups. Verapamil and nifedipine, selective blockers of L-type Ca^{2+} channels, caused dramatic inhibitions on contractions induced by ethanol in the isolated gastric fundal strips of mice. The Ca^{2+}-free medium nearly eliminated these contractions. These results suggest that extracellular Ca^{2+} and voltage-dependent L-type calcium channels may have important roles in ethanol-induced contractions in the related tissue. A similar suggestion was advanced by Zhang *et al*[11], who suggested that extracellular Ca^{2+} is necessary for ethanol to induce contractions[11]. In addition, Tirapelli *et al* also suggest that the enhanced vascular response to phenylephrine in the aortas of ethanol-treated rats is maintained by an increased extracellular Ca^{2+} influx[12]. The results of Walter and Messing are in line with our data[13]. In that study, it was reported that exposure to ethanol is associated with increases in functional L-type channels[13]. The experimental results of a study by Altura *et al* also confirm our data[14]. In that study, the authors reported that ethanol produced contractile responses in rat cerebral arterioles and venules, and that two selective blockers of L-type calcium channels, nimodipine and verapamil, inhibited the alcohol-induced cerebrovasospasms[14]. In another study, the role of L-type Ca^{2+} channels was emphasized in the effects of ethanol on rat fetal hypothalamic cells in culture[15]. In addition, the results of a study by Lombardi *et al* reported data that aligns with our hypothesis[16]. In that study, nifedipine inhibited the bronchoconstriction induced by ethanol in normal healthy volunteers. In our study, it seems that ethanol would directly activate the L-type calcium channels of smooth muscle cells in the mouse gastric fundus. Since ethanol also has a nonpolar character, it is likely that the binding site of ethanol is a hydrophobic site on the channel. Further work is needed to clarify this point. A similar suggestion was made by Oz *et al*[4], who suggest that ethanol directly binds to voltage-dependent calcium channels. They also reported that the binding site of ethanol is a hydrophobic pocket on the channel protein[4]. The results of a study by Simasko *et al* also confirm our suggestion. In that study, it was reported that ethanol could directly modulate cytoplasmic calcium levels in hypothalamic cells, mostly by a pathway that involves extracellular Ca^{2+} and voltage-dependent calcium channels[15].

To clarify the role of intracellular Ca^{2+} on ethanol-induced contractions, we first used two specific antagonists of ryanodine receptors: ryanodine and ruthenium red. Ryanodine receptors (RyRs) form a class of intracellular calcium channels present in various forms of excitable animal tissues such as muscles and neurons. It is the major cellular mediator of calcium-induced calcium release in animal cells. RyRs mediate the release of calcium ions from the sarcoplasmic/endoplasmic reticulum, an essential step in muscle contraction. Ryanodine and ruthenium red are the specific antagonists of RyRs[17]. Ryanodine locks the RyRs at a half-open state at nanomolar concentrations, yet fully closes them at micromolar concentrations[18]. In our study, micromolar concentrations of ryanodine and ruthenium red significantly inhibited the contractions induced by ethanol in isolated gastric fundal strips of mice. This result suggests that RyRs, in other words, intracellular Ca^{2+}, may have a impact on contractions induced by ethanol in the gastric fundal strips. The results of a study conducted by Oba *et al* confirm our results[19], reporting that ethanol potentiates the Ca^{2+}-induced Ca^{2+} release mechanism, which is related to RyRs[19].

In our study, we also used cyclopiazonic acid (CPA), a selective inhibitor of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA), to clarify the role of intracellular Ca^{2+} on ethanol-induced contractions in the gastric fundus. SERCA plays a central role in muscle contractility[20]. SERCA actively transports Ca^{2+} into the SR and regulates cytosolic Ca^{2+} concentration, SR Ca^{2+} load, and the rate of contraction and relaxation of the muscle[20]. In the present study, CPA significantly inhibited ($P<0.05$) the contractions induced by ethanol in isolated mouse gastric fundal strips in a concentration-dependent manner. This result suggests a role for intracellular Ca^{2+} in contractions induced by ethanol and corroborates the results found with ryanodine and ruthenium red.

In the present study, we also used caffeine, a depleting agent of intracellular Ca^{2+} stores, to confirm the role of intracellular Ca^{2+} stores in ethanol contractions. Caffeine is a Ca^{2+}-releasing agent that affects intracellular calcium stores. It activates RyRs by potentiating sensitivity to native ligand Ca^{2+}, then it depletes intracellular Ca^{2+} stores[18, 21]. In this study, caffeine significantly inhibited ($P<0.05$) the contractile responses to ethanol in the isolated gastric fundal strips of mice. This result supports a role for intracellular Ca^{2+}. The results of a study

by Werber *et al* support our hypothesis[2]. In that study, the authors reported that ethanol evokes vasoconstriction in rat aortas, mediated by caffeine-sensitive pools. In addition, the combination of caffeine plus CPA potentiated the inhibitory action of caffeine on ethanol-induced contractions.

Furthermore, we also tested the combinations of ryanodine +CPA and ryanodine+CPA+caffeine. The combination of ryanodine+CPA potentiated the inhibitory action of ryanodine and CPA on ethanol-induced contractions. The combination of ryanodine+CPA+caffeine completely inhibited the contractile responses to ethanol in gastric fundal strips of mice. This finding further confirms the impact of intracellular Ca^{2+} on ethanol-induced contractions in the gastric fundal strips of mice. The results of a study made by Zhang *et al* are in accord with our suggestion[22]. In that study, the authors reported that treatment of smooth muscle cells with ethanol resulted in an elevation in intracellular Ca^{2+}. The results suggest that intracellular Ca^{2+} may also have an important role in contractile responses induced by ethanol in the gastric fundus.

Overall, the data of the present study could be used to suggest that both extracellular and intracellular Ca^{2+} have important roles in regulating ethanol-induced contractions in the gastric fundus of the mouse.

Acknowledgements

This study was supported by the Scientific and Technical Research Council of Turkey (TUBITAK) (SBAG-HD-244, 107S290). Part of this work was presented at VII National Congress of Neuroscience, Adana, Turkey, 2008.

Author contribution

Naciye YAKTUBAY DÖNDAŞ designed the research; Mahir KAPLAN, Derya KAYA, Naciye YAKTUBAY DÖNDAŞ performed the research; Naciye YAKTUBAY DÖNDAŞ, Ergin ŞİNGİRİK analyzed the data; Naciye YAKTUBAY DÖNDAŞ wrote the paper.

References

1 Belia S, Mannucci R, Lisciareli M, Cacchio M, Fano G. Double effect of ethanol on intracellular Ca^{2+} levels in undifferentiated PC12 cells. Cell Signal 1995; 7: 389–95.

2 Werber AH, Morgan RA, Zhou P, Yang C. Intracellular mechanisms of constriction of rat aorta by ethanol. Alcohol 1997; 14: 351–60.

3 Cofan M, Nicolas JM, Fernandez-Sola J, Robert J, Tobias E, Sacanella E, *et al*. Acute ethanol treatment decreases intracellular calcium-ion transients in mouse single skeletal muscle fibres *in vitro*. Alcohol Alcohol 2000; 35: 134–8.

4 Oz M, Tchugunova YB, Dunn SMJ. Direct inhibition of voltage-dependent Ca^{2+} fluxes by ethanol and higher alcohols in rabbit T-tubule membranes. Eur J Pharmacol 2001; 418: 169–76.

5 Cooke AR, Birchall A. Absorption of ethanol from the stomach. Gastroenterology 1969; 57: 269–72.

6 Sim SS, Choi JC, Min DS, Rhie DJ, Yoon SH, Hahn SJ, *et al*. The involvement of phospholipase A2 in ethanol-induced gastric muscle contraction. Eur J Pharmacol 2001; 413: 281–5.

7 Sonoyama K, Tajima K, Fujiwara R, Kasai T. Intravenous infusion of hexamethonium and atropine but not propranolol diminishes apolipoprotein A-IV gene expression in rat ileum. J Nutrit 2000; 130: 637–41.

8 Catterall WA. Molecular mechanisms of gating and drug block of sodium channels. Novartis Found Symp 2002; 241: 206–32.

9 Keshavarzian A, Muska B, Sundaresan R, Urban G, Fields J. Ethanol at pharmacologically relevant concentrations inhibits contractility of isolated smooth muscle cells of cat esophagus. Alcohol Clin Exp Res 1996; 20: 180–4.

10 Sim SS, Choi JC, Min DS, Rhie DJ, Yoon SH, Hahn S, *et al*. Effect of ethanol on spontaneous phasic contractions of cat gastric smooth muscle. Scandinavian J Gastroenterol 2002; 37: 23–7.

11 Zhang A, Altura BT, Altura BM. Ethanol-induced contraction of cerebral arteries in diverse mammals and its mechanism of action. Eur J Pharmacol 1993; 248: 229–36.

12 Tirapelli CR, Al-Khoury J, Bkaily G, D'Orleans-Juste P, Lanchote VL, Uyemura SA, *et al*. Chronic ethanol consumption enhances phenylephrine-induced contraction in the isolated rat aorta. J Pharmacol Exp Ther 2006; 316: 233–41.

13 Walter HJ, Messing RO. Regulation of neuronal voltage-gated calcium channels by ethanol. Neurochem Int 1999; 35: 95–101.

14 Altura BM, Altura BT, Gebrewold A. Alcohol-induced spasms of cerebral blood vessels: relation to cerebrovascular accidents and sudden death. Science 1983; 220: 331–3.

15 Simasko SM, Boyadjieva N, De A, Sarkar DK. Effect of ethanol on calcium regulation in rat fetal hypothalamic cells in culture. Brain Res 1999; 824: 89–96.

16 Lombardi C, Spedini C, Govoni S. Effect of calcium entry blockade on ethanol-induced changes in bronchomotor tone. Eur J Clin Pharmacol 1985; 28: 221–2.

17 Vites A, Pappano A. Distinct modes of inhibition by ruthenium red and ryanodine of calcium-induced calcium release in avian atrium. J Pharmacol Exp Ther 1994; 268: 1476–84.

18 Xu L, Tripathy A, Pasek D, Meissner G. Potential for pharmacology of ryanodine receptor/calcium release channel. Ann N Y Acad Sci 1998; 853: 130–48.

19 Oba T, Ishikawa T, Murayama T, Ogawa Y, Yamaguchi M. H_2O_2 and ethanol act synergistically to gate ryanodine receptor/calcium-release channel. Am J Physiol Cell Physiol 2000; 279: 1366–74.

20 Periasamy M, Bhupathy P, Babu GJ. Regulation of sarcoplasmic reticulum Ca^{2+} ATPase pump expression and its relevance to cardiac muscle physiology and pathology. Cardiovasc Res 2008; 77: 265–73.

21 Reeve HL, Archer SL, Soper M, Weir EK. Dexfenfluramine increases pulmonary artery smooth muscle intracellular Ca^{2+}, independent of membrane potential. Am J Physiol Lung Cell Mol Physiol 1999; 277: 662–6.

22 Zhang A, Cheng TPO, Altura BT, Altura BM. Chronic treatment of cultured cerebral vascular smooth muscle cells with low concentration of ethanol elevates intracellular calcium and potentiates prostanoid-induced rises in $[Ca^{2+}]_i$: relation to etiology of alcohol-induced stroke. Alcohol 1997; 14: 367–71.