

Contribution of phospholipase C and protein kinase C but not endothelin-converting enzyme to contractile responses of ethanol

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Aim: To evaluate the possible roles of phospholipase C, protein kinase C, and endothelin-converting enzyme on contractions induced by ethanol (164 mM) in isolated gastric fundal strips of mice.

Materials and methods: After the mice were killed, the stomach of each was removed and longitudinal muscle strips were prepared from the gastric fundus. The strips were mounted in organ baths and their responses were recorded isometrically. Ethanol (164 mM) was added to the organ baths and a steady-state contraction was obtained. After the first ethanol response was recorded, the preparations were incubated separately with lidocaine (1-100 µM), neomycin (10-500 µM), safinol (0.5-5 µM), and SM-19712 (1-50 µM) for 40 min. The second ethanol response was then examined in the presence of the drug used in the incubation period.

Results: Lidocaine (1-100 µM), a local anesthetic agent, did not modulate the contractions induced by ethanol in isolated gastric fundal strips of mice. In contrast, neomycin (10-500 µM), a selective inhibitor of phospholipase C, and safinol (0.5-5 µM), a selective inhibitor of protein kinase C, decreased the ethanol-induced contractions. SM-19712 (1-50 µM), a selective inhibitor of endothelin-converting enzyme, failed to affect these contractions.

Conclusion: The contractile effect of ethanol may be muscular rather than neuronal in the gastric fundal strips of mice. In addition, phospholipase C and protein kinase C pathways may have a role in contractions due to ethanol in the mouse gastric fundus. On the other hand, endothelin-converting enzyme may not have a regulatory role in the contractile responses of ethanol in the same tissue.

Key words: Ethanol, contraction, signaling, phospholipase C, protein kinase C, endothelin-converting enzyme

Etanolün kontraktıl cevaplarında endotelin dönüştürücü enzimin değil, fosfolipaz C ve proteinkinaz C'nin katkısı

Amaç: İzole fare gastrik fundus striplerinde etanol (164 mM) ile indüklenen kontraksiyonlarda fosfolipaz C, protein kinaz C ve endotelin dönüştürücü enzimin muhtemel rolünü araştırmak.

Yöntem ve gereç: Fareler öldürüldükten sonra mide izole edildi ve longitudinal kas stripleri gastrik fundustan hazırlandı. Stripler organ banyosuna asıldı ve cevaplar izometrik olarak kaydedildi. Etanol (164 mM) organ banyolarına uygulandı ve stabil kontraksiyon cevabı elde edildi. İlk etanol cevabı kaydedildikten sonra, preparatlar ayrı ayrı olarak lidokain (1-100 µM), neomisin (10-500 µM), safinol (0.5-5 µM) ve SM-19712 (1-50 µM) ile 40 dakika boyunca inkübe edildi. Daha sonra aynı kimyasal ajan varlığında ikinci etanol cevabı elde edildi.

Bulgular: Lidokain (1-100 µM) (lokal anestezi ajan) izole fare gastrik fundal striplerinde etanol ile indüklenen kontraksiyonları modüle etmedi. Bunun zıtında, neomisin (10-500 µM) (fosfolipaz C'nin selektif inhibitörü) ve safinol (0.5-5 µM) (protein kinaz C'nin selektif inhibitörü) etanol ile indüklenen kontraksiyonları azalttı. Bununla birlikte, SM-19712 (1-50 µM) (endotelin dönüştürücü enzimin selektif inhibitörü) bu kontraksiyonları etkilemedi.

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Sonuç: Fare gastrik fundal striplerinde etanolün kontraktil cevapları nöronal olmaktan çok müküler olabilir. Buna ilave olarak, fare gastrik fundusunda etanole bağlı olan kontraksiyonlarda fosfolipaz C ve protein kinaz C yollarının rolü olabilir. Diğer taraftan, aynı dokuda etanolün kontraktil cevaplarında endotelin dönüştürücü enzimin regülatör rolü olmayabilir.

Anahtar sözcükler: Etanol, kasılma, signaling, fosfolipaz C, protein kinaz C, endotelin dönüştürücü enzim

Introduction

The recent recognition of an association between ethanol and toxicity has led to an increased interest in clarifying the molecular mechanism of the effect of ethanol in biological systems. Ethanol may have ischemic and hemorrhagic actions in the brain. In addition, alcohol (ethanol) consumption results in a significant increase in the morbidity of the gastrointestinal tract (1). It also facilitates the development of gastroesophageal reflux disease and gastritis (2). Furthermore, chronic ethanol consumption leads to gastrointestinal carcinogenesis (3). Since ethanol has complex effects on putative proteins in the cell, it is difficult to clarify the molecular mechanism of its effect in biological systems. It has been reported that the function of a variety of receptors and channels may be altered by ethanol (4,5). Recently it was suggested that ethanol has a role in the contractile mechanism of muscle (6,7). Depending on the tissue and/or dose of ethanol used in experiments, it can contract smooth muscle (6,8,9), but the role of signal transduction in the contractile mechanism of ethanol is not yet fully understood. Therefore, in the present study, we aimed to clarify the molecular mechanism that contributes to contractions induced by ethanol in the gastric smooth muscle. To do this, we first planned to clarify whether the contractile effect of ethanol is due to muscular or neuronal mechanisms in the smooth muscle. Lidocaine, a local anesthetic agent, was used to clarify this point. Since signal transductions, such as phospholipase C (PLC) and protein kinase C (PKC), interfere with excitation-contraction coupling in the smooth muscle and have a role in intracellular signaling pathways (6,10), we evaluated the possible role of PLC and PKC on the contractile responses of ethanol in the gastric fundus. Moreover, according to our knowledge, there is no study in the literature investigating the role of endothelin-converting enzyme (ECE) on the contractile responses of ethanol

in the gastric fundus. Therefore, we investigated the possible role of ECE in the contractile responses of ethanol in the gastric fundal strips of mice. The present study reports the role of PLC, PKC, and ECE in contractions induced by ethanol (164 mM) in the gastric fundal strips of mice. This research contributes to the understanding of the molecular mechanisms of contractions induced by ethanol in the gastric fundus. It may also contribute to the development of drug research aimed to prevent the toxic effects of ethanol in the stomach.

Materials and methods

Animals

Swiss albino mice of either sex, weighing 20-25 g, were purchased from the Medical Sciences Experimental Research and Application Center of Çukurova University. Roughly equal numbers of each sex were used throughout the experimental groups. The experimental procedures were approved by the Animal Care Committee of Çukurova University (TIBDAM) and the experiments were carried out in accordance with *Principles of Laboratory Animal Care* (National Institutes of Health guidelines; publication no. 86-23, revised 1984). All animals were kept under standard laboratory conditions (12 h dark and 12 h light).

Tissue preparation

Mice were fasted for 24 h with free access to water. They were killed by stunning and cervical dislocation. The stomach was removed and longitudinal muscle strips (approximately 15 mm long × 3 mm wide) were prepared from the gastric fundus (1 gastric fundal muscle strip from each animal was used). The strips were then mounted under a resting tension of 0.5 g in 10 mL of organ baths containing Tyrode's solution (mM: NaCl 136.7, KCl 2.6, CaCl₂ 1.8, MgCl₂·6H₂O 0.95, NaH₂PO₄·2H₂O 0.41, NaHCO₃ 11.9, and

glucose 5.05). The bath medium was maintained at 37 °C and bubbled with 95% O₂ and 5% CO₂. 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; Commat, Ankara, Turkey). Data were recorded and stored using data acquisition software (MP35 System, BIOPAC, Goleta, CA, USA).

Experimental protocols

After a preincubation period of 1 h, the basal tonus of the preparation was recorded for 5 min and then ethanol (164 mM) was added to the organ baths. This process 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 incubation, the same protocol was repeated on the same preparation. These experiments were assessed as a general control group.

In other experimental groups, after the first ethanol (164 mM) response was recorded and tissue was rinsed with Tyrode's solution, the preparations were incubated separately with lidocaine (1, 10, and 100 µM), neomycin (10, 100, and 500 µM), safinol (0.5, 1, and 5 µM), and SM-19712 (1, 5, 10, and 50 µM). After a 40-min incubation, the second ethanol (164 mM) response was examined in the presence of the drug used in the incubation period. Each concentration of substances was tried on a separate group (n = 4-8).

Drugs

Ethanol anhydrous, lidocaine, neomycin sulfate, safinol, and SM-19712 (4-chloro-N-[[[4-cyano-3-methyl-1-phenyl-1H-pyrazol-5-yl]amino]carbonyl] benzenesulfonamide monosodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of lidocaine, SM-19712, and neomycin sulfate were dissolved in distilled water. Safingol was dissolved in dimethyl sulfoxide (DMSO; 1/10,000 dilution).

Statistical analysis

In this study, 2 contractile responses due to ethanol were obtained in each experiment. The first contractile response was obtained in the absence of the drug (it was presumed to be a control response and regarded as 100%). The second contractile response was

obtained in the presence of the drug. The second contractile response due to ethanol was calculated as a percentage of the response of the control (the first response) in the same tissue. For each experimental group, the results were represented as mean ± standard error of the mean (SEM) and statistically compared with the general control group (in the general control group, the first and second contractile responses to ethanol were obtained in the absence of the drug). The program InStat (GraphPad Software) was used for statistical analysis. One-way analysis of variance (ANOVA) was performed followed by a Bonferroni post hoc test. A value of P < 0.05 was considered statistically significant.

Results

Ethanol (164 mM) produced reproducible contractions in gastric fundal strips of mice (Figures 1 and 2, Table 1). Lidocaine (1-100 µM), a ganglionic blocking agent, failed to affect the contractile responses induced by 164 mM ethanol in isolated gastric fundal strips of mice (Table 2). Neomycin (10, 100, and 500 µM), a PLC inhibitor, significantly inhibited the contractile responses induced by ethanol in a concentration-dependent manner (Figure 3). Whereas the lower concentrations (0.5 and 1 µM) of safinol, a selective inhibitor of PKC, nonsignificantly inhibited the contractile responses of ethanol (Figure 4), the highest concentration (5 µM) of this agent significantly inhibited these contractile responses (Figure 4). SM-19712 (1, 5, 10, and 50 µM), a selective inhibitor of ECE, did not affect the contractile responses induced by ethanol in the gastric fundal strips of mice (Table 3).

Discussion

The concentration of ethanol used in the present study was 164 mM (1 v/v %). We did not think that this concentration was too high for the gastric fundus. The experimental results of a study done by Cooke and Birchall (11) support our idea. Those authors reported that the gastric muscle is exposed to concentrations higher than that found in blood. In addition, Sim et al. (12) also reported that 342 mM (2 v/v %) ethanol used in isolated cat gastric fundus appeared to be attainable in the stomach muscle

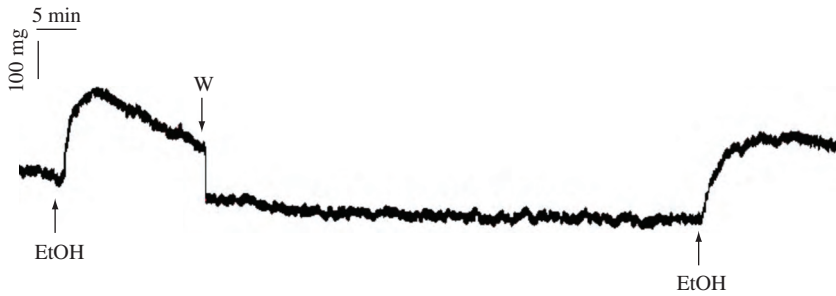


Figure 1. A representative trace showing reproducible contractions induced by 164 mM ethanol (general control) in isolated gastric fundal strips of mice. EtOH: ethanol, W: wash.

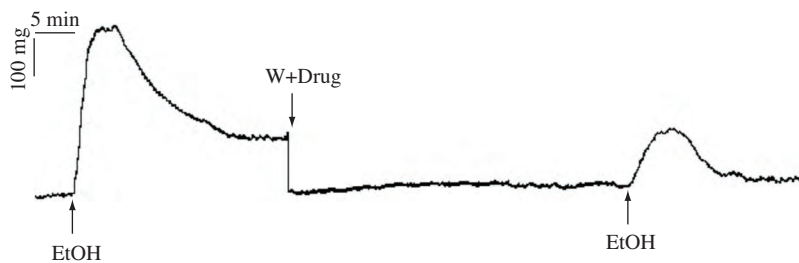


Figure 2. A representative trace showing the effects of the drugs used to antagonize the contractile effect of ethanol in isolated gastric fundal strips of mice. EtOH: ethanol, W: wash.

Table 1. The effects of ethanol (EtOH) on isolated gastric fundal strips of mice. For each group, data are means \pm SEM of gastric fundal strips from 8 animals.

Group	First application of 164 mM EtOH (mg)	Second application of 164 mM EtOH (mg)
Control	118.6 \pm 10.3	135.7 \pm 12.9
DMSO control (1/10,000 dilution)	115.8 \pm 12.9	135.5 \pm 14.8

Table 2. The effects of lidocaine (1-100 μ M) on contractions induced by EtOH (164 mM) in isolated gastric fundal strips of mice. For each concentration of the drug, data are means \pm SEM of gastric fundal strips from 4 to 6 animals.

Drug name	Drug Concentration (μ M)	EtOH (% contraction)	EtOH + drug (% contraction)
Lidocaine	1	114.4 \pm 11.6	113.9.1 \pm 13.8
	10	114.4 \pm 11.6	115.6 \pm 12.9
	100	114.4 \pm 11.6	114.9 \pm 14.4

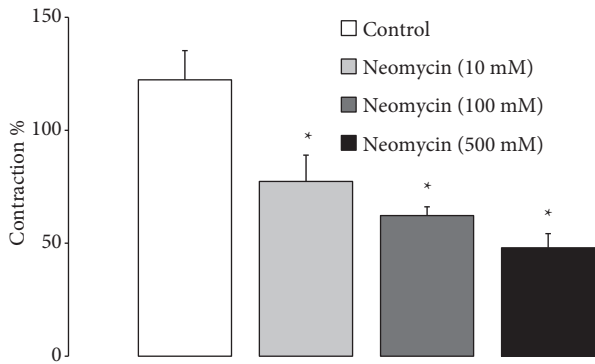


Figure 3. Effect of neomycin (10-500 µM) on contractions induced by ethanol (164 mM) in isolated gastric fundal strips of mice. For each concentration of the drug, data are means ± SEM of gastric fundal strips from 6-8 animals. Control: 164 mM ethanol. Significant differences were evaluated by one-way analysis of variance (ANOVA). Post hoc: Bonferroni. *P < 0.05 versus control.

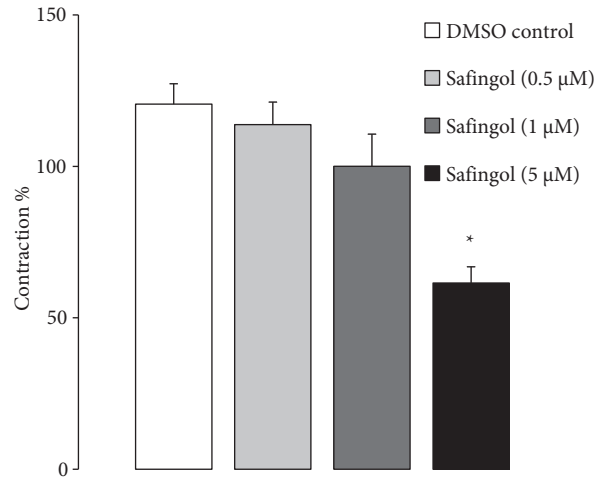


Figure 4. Effect of safingol (0.5-5 µM) on contractions induced by ethanol (164 mM) in isolated gastric fundal strips of mice. For each concentration of the drug, data are means ± SEM of gastric fundal strips from 4 to 8 animals. Control: 164 mM ethanol including DMSO (1:10,000 dilution). Significant differences were evaluated by one-way analysis of variance (ANOVA). Post hoc: Bonferroni. *P < 0.05 versus control.

Table 3. Effect of SM-19712 (1-50 µM) on contractions induced by EtOH (164 mM) in the mouse gastric fundal strips. For each concentration of the drug, data are means ± SEM of gastric fundal strips from 5 animals.

Drug name	Concentration (µM)	164 mM EtOH (% contraction)	164 mM EtOH + Drug (% contraction)
SM-19712	1	114.4 ± 11.6	109.6 ± 11.9
	5	114.4 ± 11.6	117.6 ± 9.8
	10	114.4 ± 11.6	106.2 ± 15.2
	50	114.4 ± 11.6	112.6 ± 12.5

tissues of living animals. In light of this knowledge, we can suggest that the ethanol concentration used in the present study was acceptable for the gastric fundus.

Lidocaine is a local anesthetic agent. It alters depolarization in neurons by blocking the fast voltage-gated sodium (Na⁺) channels in the cell membrane (13). With a sufficient blockade, the membrane of the presynaptic neuron will not depolarize and thus fails to transmit an action potential. It has a high degree of selectivity in the blockage of neurons and neuron signaling. In this study, lidocaine failed to

affect the contractile responses induced by ethanol in the mouse gastric fundus. This observation suggests that ethanol may directly affect the muscle cells of the mouse gastric fundus. Similar results were obtained by Keshavarzian et al. (14) in cat esophagus. In that study, it was reported that because the response to ethanol was not abolished in cats by cervical vagotomy or intravenous tetrodotoxin, they surmised a direct effect of ethanol on the muscle cells (14).

Since drugs activating PLC are generally known to elicit smooth muscle contractions, we investigated whether ethanol-induced contractions were mediated

through the activation of PLC. Phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate the signaling molecules diacylglycerol and inositol 1,4,5-triphosphate, leading to calcium mobilization and contraction (10). In the present study, neomycin, a selective inhibitor of PLC (15), significantly inhibited the contractile response of ethanol in gastric fundal strips of mice. This result suggests that the PLC pathway may be involved in ethanol-induced gastric smooth muscle contraction via regulation of intracellular Ca²⁺ levels. Katsura et al. (16) reported that ethanol inhibits the activity of PIP₂-specific PLC and activates cytosolic PLC in the mouse brain. In addition, Higashi and Hoek (17) suggested that ethanol causes desensitization of the receptor-mediated PLC secondary to the ethanol-induced activation of PLC and the activation of PKC. Recently, phosphatidylinositol (3,4,5)-triphosphate (PIP₃) has received more attention because it has been suggested to act as a second messenger (18). However, there is no direct evidence that PIP₃ is associated with ethanol-induced contractions. Further work is needed to clarify this point.

Multiple signaling pathways may participate in the mechanisms of contractions induced by ethanol in the gastric fundus. It was indicated that an increment in intracellular Ca²⁺ concentration occurs when muscle cells are exposed to ethanol (19). This [Ca²⁺]_i increment could occur by a direct interaction with the contractile proteins and/or the effects of Ca²⁺ acting as a cofactor for some cellular signal pathway(s), such as PKC (20). PKC, a family of Ca²⁺-sensitive and Ca²⁺-insensitive phospholipid-dependent protein kinases, has been shown to play an important role in cellular signal transduction. Several observations raise the possibility that activation of PKC isoforms might be involved in the vasoconstriction induced by ethanol (6,21). However, there is no direct evidence that PKC is associated with ethanol-induced gastric smooth muscle contraction. With these points in mind, we designed the present study to determine whether safingol, a selective inhibitor of PKC, would inhibit the contractile responses of ethanol in the gastric fundal strips of mice. Safingol is a saturated analog of sphingosine, the naturally occurring lysosphingolipid. PKC inhibition by lysosphingolipids results from competitive interaction with the regulatory diacylglycerol/phorbol-binding domain of

PKC (22). In the present study, safingol significantly inhibited the contractions induced by ethanol in the gastric fundal strips of mice. This result suggests that PKC may have a role in contraction due to ethanol in the related tissue. The experimental results obtained by Werber et al. (23) are in accordance with our findings. Those authors suggest that PKC may have a role in ethanol-induced vasoconstriction in the rat aorta. Similarly, Jover et al. (21) also confirmed our hypothesis. They reported the inhibitory effects of PKC inhibitors on ethanol-induced contractions in isolated rat aorta (21). These signal transductions may have a role in ethanol-induced contractions by using intra- and/or extracellular Ca⁺⁺. Our recent study supports our hypothesis (8). In that study, we showed that intra- and extracellular Ca⁺⁺ have important roles in contractions induced by ethanol in the mouse gastric fundus (8). In contrast to our recent study, Jakupaj et al. (24) reported that ethanol provokes contractions in the pulmonary artery smooth muscle of dogs; in this response, neither intra- nor extracellular Ca⁺⁺ plays any role. The cause of these differences may be explained by the different tissues of different animals used in the experiments and/or different experimental conditions.

In the present study, we also evaluated the possible role of ECE on contractions induced by ethanol in the mouse gastric fundus. ECE is a critical enzyme that is involved in endothelin-1 (ET-1) synthesis (25). It is well known that there are at least 3 known endothelin receptors, AT_A, ET_{B1}, and ET_{B2}, all of which are G protein-coupled receptors whose activation results in elevation of intracellular free calcium (26). It is known that ET-1 has important actions for gastrointestinal smooth muscle motility, but its precise mechanism remains unsolved (27). In the gastrointestinal tract, ET-1 causes contraction of the stomach (28). Most actions of ET-1 occur via its direct action on the smooth muscle (29). According to our knowledge, this is the first report evaluating the role of ET-1 on contractions due to ethanol in the gastric fundus. To clarify the role of ET-1 on these contractions, we used SM-19712, a selective inhibitor of ECE. In all of the concentrations that we used (1-50 μM), SM-19712 failed to affect the contractions due to ethanol in the gastric fundal strips of mice. We think that the concentrations of SM-19712 used in this study were high enough to inhibit the effect

of ECE in the mouse gastric fundus, and the results of a study done by Umekawa et al. (30) confirm this. In that study, it was reported that SM-19712 inhibited ECE solubilized from rat lung microsomes with a half-maximal inhibitory concentration value of 42 nM and, at 10-100 µM, had no effect on other metalloproteases such as neutral endopeptidase and angiotensin-converting enzyme, showing a high specificity for ECE (30). The experimental results of the present study suggest that ET-1 may not have a role on contractions induced by ethanol in the mouse gastric fundus. In contrast to our results, Tirapelli et al. (7) suggested that ethanol intake enhanced the ET-1-induced contractile response of endothelium-intact but not endothelium-denuded rat carotid rings. In that study, the authors suggested that because of the existence of both ET_A and ET_B vasoconstrictor receptors located on the smooth muscle and vasorelaxant endothelial ET_B receptors located on endothelium, the potentiation of ET-1-

induced vascular reactivity was due to the reduced expression of relaxing endothelial ET_B receptors in the rat carotid artery (7). The reason for the differences between studies might be explained by the different tissues used in the experiments.

Overall, the data of the present study could be used to suggest that the contractile effect of ethanol in the mouse gastric fundus may be directly due to its muscular effect rather than a neuronal effect. In addition, PLC and PKC pathways may have a role in contractions induced by ethanol in the mouse gastric fundus. However, ET-1 may not have a regulatory role in ethanol-induced contractile responses in the same tissue.

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