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The involvement of phospholipase A_2 in ethanol-induced gastric muscle contraction

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Abstract

To understand the underlying mechanism of ethanol in tonic contraction, the effect of ethanol on phospholipase A_2 and phospholipase C activities and the effects of phospholipase inhibitors on ethanol-induced contraction of cat gastric smooth muscle were tested. Circular muscle strips (2.0×0.2 cm) obtained from the fundus of cat stomach were used to measure isometric contraction. Ethanol elicited tonic contraction and activated phospholipase A_2 activity in a dose-dependent manner. Phospholipase A_2 inhibitors, manoalide (0.1–10 μM) and oleyloxyethyl phosphorylcholine (1–10 μM), significantly inhibited ethanol-induced contraction. Furthermore, 342 mM ethanol-induced contraction was significantly inhibited by cyclooxygenase inhibitors, ibuprofen (10–100 μM) and indomethacin (10–100 μM), but not by lipoxygenase inhibitors. On the other hand, phospholipase C inhibitors had no effect on ethanol-induced contraction, indicating that phospholipase C is not involved in ethanol-induced contraction. It is suggested from the above results that ethanol-induced contraction in cat gastric smooth muscle is, in part, mediated by phospholipase A_2 and cyclooxygenase pathways.

Keywords: Cyclooxygenase; Ethanol; Lipoxygenase; Phospholipase A_2; Phospholipase C; Smooth muscle

1. Introduction

Although ethanol has diverse biological activities in a variety of tissues, such as the brain, vessels, pancreas, liver and gastrointestinal tract (Cooke, 1972; Imamura et al., 1985; Nakamura et al., 1991; Singh et al., 1997; Tachibana et al., 1996), the underlying mechanism of ethanol still remains unclear. Ethanol has dual effects on gastrointestinal motility, depending on its concentration. Ethanol at low concentrations inhibits the amplitude and frequency of phasic contractions in canine antral smooth muscle (Sanders and Bauer, 1982; Sanders and Berry, 1985) and inhibits esophageal contractility in cats, the mechanism of which may be the inhibition of extracellular Ca^{2+} influx (Keshavarzian et al., 1994). High concentrations of ethanol cause tonic contraction of vascular smooth muscle in rats and of gastric smooth muscle in guinea pigs. It was reported that the contractile mechanism of ethanol was, in part, mediated by protein kinase C and tyrosine kinase (Werber et al., 1997; Zheng et al., 1997).

There is little evidence that ethanol has an influence on signal pathways. It has been reported that ethanol suppresses muscarinic receptor-operated signal transduction, through the phospholipase C pathway in neuroblastoma SH-SY5Y cells (Larsson et al., 1998). In the hippocampus and medial frontal cortex of rats, ethanol inhibits the activities of phospholipase C and phospholipase A_2 by 20–30% and 25%, respectively (Allan et al., 1997). However, there have been no studies examining the effect of ethanol on signal pathways in gastric smooth muscle.

Thus, to investigate the underlying mechanism of ethanol in tonic contraction, we examined the effect of ethanol on phospholipase A_2 and phospholipase C activi-

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ties, and the effects of phospholipase inhibitors on ethanol-induced contraction of cat gastric smooth muscle.

2. Materials and methods

2.1. Drugs

Ibuprofen, indomethacin, caffeic acid, baicalein, manoalide, oleyloxyethyl phosphorylcholine, U73122 and neomycin were obtained from Sigma (St. Louis, MO, USA); [^{3}H]inositol was from New England Nuclear (Boston, MA, USA); 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (10-pyren PC) was from Molecular Probes (Eugene, OR, USA); ethanol was from Merck (Darmstadt, Germany). All reagents used were of analytical grade.

2.2. Preparation of gastric smooth muscle strips

The study protocol complies with the European Community guidelines for the use of experimental animals and was approved by the Committee on the Use of Animal of the Catholic University of Korea. Cats of either sex (2.2–3.5 kg) were anesthetized with 20% urethane (5 ml/kg, i.p.) following 16 h of fasting, but with water, ad libitum. The whole stomach was removed from each cat, and the mucous membrane was peeled off in ice-cold Krebs bicarbonate solution (in mM: 120.8 NaCl, 4.5 KCl, 15.5 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 and 5.6 dextrose). The Krebs bicarbonate solution was aerated with 95% O_2–5% CO_2 until the pH was 7.4. Circular muscle strips (1.0×0.2 cm) were prepared from the fundus, cutting at right-angles to the greater curvature (Sim et al., 1997).

2.3. Measurement of contractile response

Circular muscle strips were used to measure contraction in a cylinder-shaped muscle chamber (10-ml capacity) filled with Krebs bicarbonate solution. The solution of the chamber was kept at 36°C and was bubbled with a mixture of 95% O_2–5% CO_2 at pH 7.4. To record isometric contraction, the lower end of the muscle preparation was anchored to a steel hook and the upper end to a force transducer (FT03, Grass Instruments, Quincy, MA, USA) connected to a Grass 7E polygraph (Baek et al., 2000). The preparation was loaded with a tension of 2 g and allowed to equilibrate with the solution for 30 min. The final concentrations of drugs used were achieved by adding 0.01 ml into the muscle chamber. Drugs were administered 4 min before treatment with ethanol. The amplitude of the contractile response was measured for 10 min and the maximal amplitude was taken as representative for each case.

2.4. Preparation of dispersed gastric smooth muscle cells

Muscle cells were isolated from muscle strips of cat stomach as described previously (Collins and Gardner, 1982). Briefly, muscle strips were dissected with a tissue slicer (Thomas, Philadelphia, PA, USA) with a 0.5-mm thickness and were digested with 0.3% collagenase, 0.3% papain and 0.03% soybean trypsin inhibitor overnight. The cells were harvested by filtration through 500 μm Nitex and then washed three times with 30 ml of enzyme-free Krebs bicarbonate solution. The cells were resuspended in an adequate volume of Krebs bicarbonate solution containing 20 mM Na-HEPES (pH 7.4) to measure the formation of inositol phosphates and phospholipase A_2 activity.

2.5. Measurement of phospholipase A2 activity

Phospholipase A_2 activity of smooth muscle cells treated with ethanol was measured by fluorometric assay (Radvanyi et al., 1989). All assays were performed in a 0.1 M Tris/HCl buffer, pH 7.5 containing 100 mM NaCl, 1 mM EDTA, 6 mM $CaCl_2$, 2 μM 10-pyren PC and 0.1% bovine serum albumin. After 10 min, fluorescence was measured, using excitation and emission wavelengths of 345 and 398 nm, respectively.

2.6. Measurement of inositol phosphates

The isolated cells were incubated in 20 ml of Krebs bicarbonate solution containing 20 mM Na-HEPES (pH 7.4), 0.1% bovine serum albumin and [^{3}H]inositol (5 μCi/ml, 25 mCi/mmol) for 180 min at 37°C. After washing twice, the cells were reconstituted in 2 ml of Krebs bicarbonate solution containing 20 mM Na-HEPES (pH 7.4), 0.1% bovine serum albumin and 10 mM LiCl. Aliquots of the resuspended cells were treated with ethanol for 10 min. The reaction was terminated by adding 1 ml of stop solution (chloroform/methanol/c-HCl = 2:1:0.01) and then 250 μl of chloroform and 250 μl of distilled water were added. After centrifugation at $2{,}000 \times g$ for 5 min, 600 μl of supernatant was loaded to a Dowex AG1X8 column, and the column was washed with 10 ml of distilled water and 20 ml of 60 mM ammonium formate/5 mM sodium borate. [^{3}H]inositol phosphates were eluted with 4 ml of 1 M ammonium formate/0.1 N formic acid. Radioactivity was determined by a liquid scintillation counter and expressed as dpm/mg of protein. Protein measurements were made to correct for differences between preparations using the bincinchoninic acid (BCA) method (Smith et al., 1985) and bovine serum albumin as standard.

2.7. Statistical analysis

The results are represented as means ± SD and analyzed statistically by analysis of variance (ANOVA); the differences between groups were determined with the Newman–Keuls test. The level of significance was set at less than 5%.

3. Results

3.1. Effects of phospholipase A2 inhibitors

Ethanol elicited tonic contraction in a dose-dependent manner (Fig. 1). Amplitude of tonic contraction by 342 mM ethanol was 0.83 ± 0.16 g, which was relatively strong compared to the contraction (2.4 ± 0.3 g) induced by the muscarinic receptor agonist, 1 μM acetylcholine. To examine whether phospholipase A_2 is involved in 342 mM ethanol-induced contraction of cat gastric smooth muscle, we used two phospholipase A_2 inhibitors, manoalide and oleyloxyethyl phosphorylcholine. Phospholipase A_2 inhibitors did not affect the basal tone of gastric smooth muscle. These inhibitors inhibited ethanol-induced contraction in a dose-dependent manner (Fig. 2). Manoalide (1 μM) and oleyloxyethyl phosphorylcholine (10 μM) significantly inhibited contraction by 38% and 31%, respectively. From these results, it is suggested that contraction by ethanol in cat gastric smooth muscle may be, in part, mediated by the phospholipase A_2 pathway.

3.2. Effects of cyclooxygenase and lipoxygenase inhibitors

Arachidonic acid, a product of phospholipid hydrolysis by phospholipase A_2, serves as a precursor of the eicosanoid complex that has various biological activities. Arachidonic acid is metabolized through the cyclooxygenase and lipoxygenase pathways. Since phospholipase

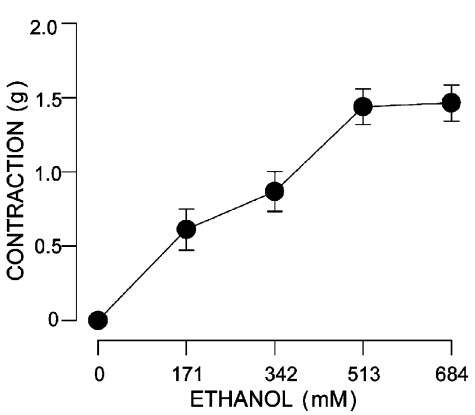

Fig. 1. Dose-response of tonic contraction to ethanol in cat gastric smooth muscle. Ethanol dose-dependently induced tonic contraction. Results are means ± SD of six separate experiments.

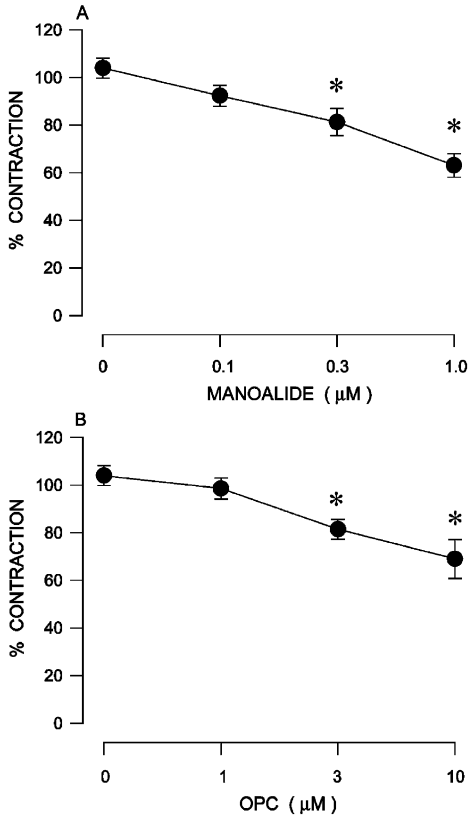

Fig. 2. Effects of phospholipase A_2 inhibitors on 342 mM ethanol-induced contraction. Manoalide (A) and oleyloxyethyl phosphorylcholine (OPC: B) were treated 4 min prior to ethanol-induced contraction, which did not have any influence on the basal tone of gastric smooth muscle. Phospholipase A_2 inhibitors inhibited the contraction in a dose-dependent manner. Results are means ± SD of six separate experiments. $^{*}P < 0.05$ significantly different from control.

A_2 inhibitors suppress ethanol-induced contraction, it is possible that cyclooxygenase and/or lipoxygenase inhibitors may affect ethanol-induced contraction. Cyclooxygenase and lipoxygenase inhibitors did not have any influence on the basal tone of gastric smooth muscle. Cyclooxygenase inhibitors, ibuprofen (100 μM) and indomethacin (100 μM), significantly inhibited ethanol-induced contraction by 40% and 75%, respectively (Fig. 3). However, lipoxygenase inhibitors, baicalein (10 μM) and caffeic acid (100 μM), only tended to inhibit contraction by 8% and 13%, respectively. These results suggest that the cyclooxygenase pathway, rather than the lipoxygenase pathway, is involved in ethanol-induced contraction.

3.3. Effects of phospholipase C inhibitors

On the whole, drugs stimulating phospholipase C elicited smooth muscle contraction. To investigate whether ethanol-induced contraction was mediated via phospholipase C, we observed the effects of phospholipase C inhibitors on ethanol-induced contraction. U73122 (10 μM) and

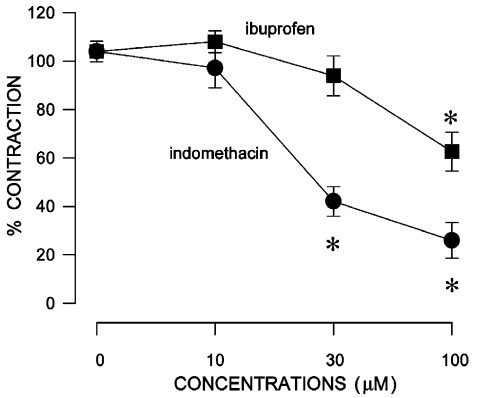

Fig. 3. Effects of cyclooxygenase inhibitors on 342 mM ethanol-induced contraction. Cyclooxygenase inhibitors were treated 4 min prior to ethanol-induced contraction, which did not have any influence on the basal tone of gastric smooth muscle. Ibuprofen and indomethacin inhibited ethanol-induced contraction in a dose-dependent manner. Results are means ± SD of six separate experiments. $^{*}P < 0.05$ significantly different from control.

neomycin (100 μM) did not have any influence on ethanol-induced contraction. However, with regards to the effect of ethanol on phospholipase C activity, ethanol at concentrations of more than 342 mM significantly inhibited the formation of [^{3}H]inositol phosphates in the resting state (Fig. 4). These results indicate that phospholipase C may not be involved in ethanol-induced contraction of cat gastric smooth muscle.

3.4. Effect of ethanol on phospholipase A2 activity

In smooth muscle contractions, phospholipase A_2 inhibitors significantly inhibited ethanol-induced contraction, suggesting that the contraction may be mediated via the activation of phospholipase A_2. To confirm the above suggestion, we measured phospholipase A_2 activity in gastric smooth muscle cells treated with ethanol. Ethanol dose-dependently stimulated phospholipase A_2 activity

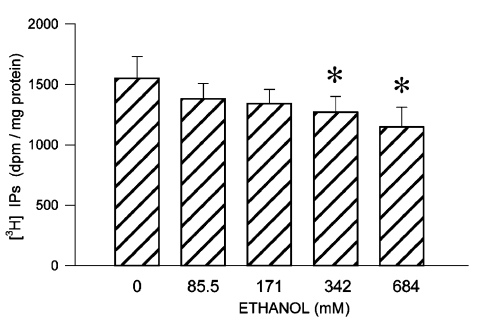

Fig. 4. Effect of ethanol on the formation of [^{3}H]inositol phosphates (Ips). Ethanol (342 mM) significantly decreased the formation of [^{3}H]inositol phosphates. Results are means ± SD of four separate experiments. $^{*}P < 0.05$ significantly different from control.

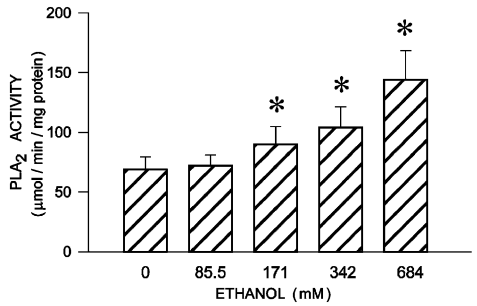

Fig. 5. Effect of ethanol on phospholipase A_2 activity. Ethanol dose-dependently stimulated phospholipase A_2 activity. Results are means ± SD of four separate experiments. $^{*}P < 0.05$ significantly different from control.

(Fig. 5). Ethanol at a concentration of 342 mM significantly increased the activity of phospholipase A_2 by 50%.

4. Discussion

The concentration of ethanol used in this study was above 0.5%, which is higher than can be achieved in blood. After the intake of 180 ml of 50% ethanol by humans, blood concentration of ethanol reached approximately 0.1% (Kaufman and Kaye, 1978). Considering that ethanol is absorbed linearly as a function of concentration across the gastric mucosa (Cooke and Birchall, 1969), it is possible that gastric muscle is exposed to concentrations higher than that found in blood. According to McGregor et al.(1990), the ethanol concentration of gastric muscle tissue reaches the range of 1.6–14.3 M, while that of serum is 0.02 M, after instilling 30 ml of 45% ethanol into the proximal stomach pouch, which was prepared surgically in dogs. These results indicate that direct diffusion from the mucosal surface possibly produces much higher concentrations of ethanol in gastric muscle than would be anticipated from serum levels. So, a 342-mM (2v/v%) concentration of ethanol used in this study does appear to be attainable in the stomach muscle tissues of living animals.

In this study, acute treatment with ethanol of cat gastric smooth muscle dose-dependently elicited tonic contraction and stimulated phospholipase A_2 activity, with phospholipase C activity being inhibited slightly. These results suggest that ethanol-induced contraction is associated with the activation of phospholipase A_2. To date, there has been little information about the effect of ethanol on phospholipase A_2 in gastric smooth muscle. Previous reports that phospholipase A_2 activity was decreased by 25% in the hippocampus and medial frontal cortex of adult rats prenatally exposed to ethanol (Allan et al., 1997) are inconsistent with our results. This discrepancy might lie in the differences in tissues used and in the duration or timing of ethanol treatment. Our results are further supported by the observation that ethanol-induced contraction was significantly inhibited by phospholipase A_2 inhibitors, manoalide and oleyloxyethyl phosphorylcholine.

Arachidonic acid resulting from phospholipid hydrolysis by phospholipase A_2 is a precursor of the eicosanoid complexes, leukotriens, thromboxanes and prostaglandins. Arachidonic acid is catabolized by the cyclooxygenase and lipoxygenase pathways. The finding that ethanol-induced contraction was significantly inhibited by phospholipase A_2 inhibitors suggests the possible involvement of cyclooxygenase/lipoxygenase pathway(s) in ethanol-induced contraction. As shown in the results, however, only the cyclooxygenase inhibitors, ibuprofen and indomethacin, significantly inhibited ethanol-induced contraction, while lipoxygenase inhibitors, baicalein and caffeic acid, did not. It has been reported that ethanol-induced contraction in the longitudinal preparation of guinea pig gastric smooth muscle was inhibited by indomethacin, but not by lipoxygenase inhibitors (Zheng et al., 1997). However, they showed that indomethacin did not inhibit ethanol-induced contraction in the circular preparation, suggesting that the contractile effect of ethanol in the longitudinal preparation is distinct from the circular preparations of guinea pig gastric smooth muscle. From the above reports and our results, the effects of cyclooxygenase inhibitors on gastric smooth muscle may be distinct from the muscle preparation and species.

Since drugs activating phospholipase C, in general, are known to elicit smooth muscle contraction, we investigated whether ethanol-induced contraction was mediated through the activation of phospholipase C. Phospholipase C inhibitors, U73122 and neomycin, did not have any influence on ethanol-induced contraction. Furthermore, ethanol inhibited phospholipase C activity indicating that ethanol-induced contraction is not mediated via the phospholipase C pathway. Such an inhibition of phospholipase C by ethanol was previously observed in other tissues (Zhang et al., 1997). Other reports implying a relationship between alcohol and the phospholipase C pathway include those that state that ethanol suppresses the muscarinic receptor-mediated phospholipase C pathway in neuroblastoma SH-SY5Y cells (Larsson et al., 1998), and that ethanol perturbes receptor-operated cytosolic free calcium concentration signals in cultured rat hepatocytes (Zhang and Farrell, 1997). In summary, the present results suggest that cyclooxygenase and phospholipase A_2 pathways are involved in ethanol-induced gastric smooth muscle contraction, but that phospholipase C is not.

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