CA\textsuperscript{2+}-ACTIVATED K\textsuperscript{+} CHANNELS INVOLVED IN DUODENAL DISMOTILITY INDUCED BY ETHANOL\textsuperscript{†}

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(Received 24 November 2006; in revised form 27 January 2007; accepted 30 March 2007; advance access publication 28 May 2007)

Abstract—The purpose of this study was to investigate the role of K\textsuperscript{+} channels in duodenal dismotility induced by ethanol in vitro. The amplitude of spontaneous contractions was reduced by ethanol in longitudinal and circular muscle, while frequency did not change. Charybdotoxin antagonized ethanol-induced inhibition of the amplitude of spontaneous contractions. Ethanol decreased ACh-induced contractions and this effect was cancelled out by charybdotoxin. Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels may be involved in duodenal dismotility induced by ethanol.

INTRODUCTION

Acute intoxication with ethanol affects many systems in the body. Gastrointestinal symptoms, such as nausea, vomiting, and diarrhoea often accompany this (Lu et al., 1997). Acute, chronic alcohol consumption affects motility, causing mucosal inflammation and cancer of the oesophagus and stomach (Franke et al., 2005b). Ethanol stimulates both gastric acid and pancreatic exocrine secretion (Cooke, 1972; Imamura et al., 1985; Tachibana et al., 1996), however, it inhibits oesophageal contractility in cats (Keshavarzian et al., 1994), and the motility of rabbit sphincter of Oddi (Sari et al., 2002). Ethanol also decreases the amplitude and frequency of phasic contractions of canine antral smooth muscle (Sanders and Bauer, 1982), and cat gastric corpus (Sim et al., 2002). Furthermore, it decreases spontaneous contractile amplitude, hyperpolarizing the resting membrane potential and the amplitude of the slow wave in canine jejunal circular smooth muscle (Lu et al., 1997).

Ethanol in low concentrations of 4 and 10\% (v/v) prolongs gastric emptying (Franke et al., 2004, 2005a). A single large dose also inhibits small bowel transit. Treatment with a large dose of alcohol for 10 days did not change gastric emptying significantly but did inhibit small intestine transit (Izbéki et al., 2001).

The cellular mechanisms which evoke effects on the gastrointestinal tract through ethanol are little known. The inhibitory effect of alcohol is partially reversed by increasing the availability of extracellular calcium (Lu et al., 1997), or by the involvement of CCK receptors (Izbéki et al., 2004) and capsaicin-sensitive neural pathways (Izbéki et al., 2002).

K\textsuperscript{+} evokes the cellular hyperpolarization and different K\textsuperscript{+} channels take part in the cellular process. As alcohol inhibits intestinal motility, we hypothesize that K\textsuperscript{+} channels could participate in this effect. The purpose of this study was to determine the role of K\textsuperscript{+} channels in the dismotility of rabbit duodenum induced by ethanol in vitro.

MATERIALS AND METHODS

Animals

Male New Zealand rabbits weighing 2–2.5 kg were maintained at a constant room temperature (22 °C) with standard rabbit fodder and free access to water. Handling, equipment used and sacrifice complied with European Council legislation 86/609/EEC on experimental animal protection. Experiment protocols were approved by the Ethics Committee of the University of Zaragoza (Spain).

Solution and substances

Krebs solution contained the following (in mM): NaCl 120, KCl 4.70, CaCl\textsubscript{2} 2.40, MgSO\textsubscript{4} 1.20, NaHCO\textsubscript{3} 24.50, KH\textsubscript{2}PO\textsubscript{4} 1.00 and glucose 5.60 at 37 °C to obtain a pH of 7.4. Ethanol was obtained from Merck (Madrid, Spain). Acetylcholine (ACh), apamin, charybdotoxin (ChTX), iberiotoxin (IbTX), glibenclamide (GB) and tetraetylammonium chloride (TEA) were purchased from Sigma (Madrid, Spain). All chemicals were analytical grade. Stock standard solution of apamin (0.2 mg ml\textsuperscript{-1}) was prepared in acetic acid and glibenclamide (0.2 mg ml\textsuperscript{-1}) in dimethyl sulphoxide (DMSO). All other drugs were dissolved in distilled water. All solutions were stored at −20 °C and fresh dilutions were made daily.

Preparation of smooth muscle segments

After 24 h of fasting, animals were humanely killed by a blow to the head. Pieces of rabbit duodenum were removed, washed, freed from mesenteric attachment and cut into smaller segments. Whole thickness segments (10 mm long and 5 mm wide) were suspended in the direction of longitudinal or circular smooth muscle fibers in a thermostatically controlled (at 37 °C) organ bath (10 ml capacity) containing Krebs solution and continuously gassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2}.
Each segment of duodenum was connected to an isometric force transducer (Pionden UF1, Graham Bell House, Camberley, UK) and stretched passively to an initial tension of 20 mN. Signal output of the mechanical activity was amplified, recorded on a computer for later analysis using the Mac Lab System/8e computer program (AD Instruments Inc., Millford MA, USA) and digitized at two samples/s per channel. Before testing, segments were allowed to equilibrate in Krebs solution for 60 min. During that time, the nutrient solution was changed every 20 min.

**Experimental protocols**

Each experimental protocol was systematically performed on four segments of longitudinal and circular smooth muscle taken from the duodenum of the same rabbit and repeated on three or four different animals. Segments that showed no spontaneous activity were discarded. The primary experimental protocol was to study the local effect of ethanol on the contractility of the duodenum. After the equilibration period, we recorded spontaneous motility for 5 min and after ACh (0.1 mM) was added to the bath. This ACh-evoked response was considered as the control. Segments of the duodenum were then incubated for 90 min with Ringer Krebs or ethanol 1, 2.3, and 3.3 µl ml⁻¹ (v/v). Once again, ACh (0.1 mM) was added to the bath and this second ACh response was compared with the control and expressed as a percentage. The second protocol was carried out to examine the role of K⁺ channels on ethanol-induced effects. We added apamin (100 nM, a blocker of small-conductance Ca²⁺-activated K⁺ channels, SKCa), charybdotoxin (10 nM, a selective blocker of intermediate- and large-conductance Ca²⁺-activated K⁺ channels, IKCa and BKCa respectively), iberiotoxin (10 nM, a non-specific K⁺ channel blocker, glibenclamide (100 nM, a blocker of ATP-sensitive K⁺ channels, KATP) or tetraethylammonium (5 mM, a non-specific K⁺ channels blocker) to the bath 15 min before incubation with ethanol (2.3 µl ml⁻¹) for 90 min.

**Data analysis**

Longitudinal and circular smooth muscle response to ACh were measured as integrated mechanical activity per second (milleinewtons per second, mN s⁻¹), and normalized per square millimeter of cross-sectional area (CSA; mm²). CSA was determined for each muscle strip using the equation: CSA (mm²) = mass (mg) [length (mm) density (mg mm⁻³)]⁻¹, where rabbit intestinal muscle density was assumed to be 1.05 mg mm⁻³. Length and mass (wet weight) of each segment were noted on completion of experiments (Reboillar et al., 2002; Grasa et al., 2006).

The amplitude and frequency of spontaneous contractions were analysed for each segment of smooth muscle. Mean amplitude (in µN) of contractions was calculated as the average of peak-to-peak differences over 5 min. The frequency of contractions was expressed as the number per minute (cpm) over a 5 min period. The amplitude and frequency of spontaneous contractions in the presence of drugs was expressed as a percentage of the values recorded in the absence of drugs (control period) before adding ACh to the bath.

Values are expressed as mean ± SEM. Means were compared using one-way variance analysis (ANOVA) tests and P-values determined using the Scheffé f-test. Differences with P-values < 0.05 were considered statistically significant.

**RESULTS**

Spontaneous contractions of longitudinal and circular smooth muscle of rabbit duodenum were phasic, with mean values of amplitude (11.6 ± 1.4 mN, N = 16 and 3.5 ± 0.8 mN, N = 18) and frequency (12.4 ± 1.5 cpm, N = 16 and 7.8 ± 1.4 cpm, N = 18) respectively.

**Effect of ethanol on spontaneous contractions**

Ethanol (1, 2.3, 3.3 µl ml⁻¹) decreased the amplitude of spontaneous contractions in longitudinal and circular smooth muscle but did not modify the frequency of spontaneous contractions in longitudinal and circular smooth muscle (Fig. 1 and Table 1).

**Effect of K⁺ channel blockers on the ethanol response in spontaneous contractions**

Apamin (100 nM), iberiotoxin (10 nM), glibenclamide (100 nM) and TEA (5 mM) did not alter the inhibition of ethanol (2.3 µl ml⁻¹) on the amplitude of spontaneous contractions in longitudinal and circular smooth muscle. Except in the case of TEA, these blockers increased the frequency of spontaneous contractions in longitudinal but not in circular smooth muscle (Table 2). ChTX (10 nM, for 90 min) did not modify the amplitude (91.1 ± 5.7 N = 13; 97.2 ± 10.4 N = 14) and the frequency (104.9 ± 2.0 N = 13; 104.9 ± 5.5 N = 13) of spontaneous contractions in longitudinal and circular smooth muscle of the duodenum respectively. ChTX (10 nM) cancelled EtOH-induced inhibition (2.3 µl ml⁻¹) of the amplitude of spontaneous contractions in longitudinal and circular smooth muscle and did not modify the frequency of spontaneous contractions in two muscles (Table 2).

**Effect of ethanol on ACh responses**

The responses of ACh (0.1 mM) did not change after 90 min of incubation with either Krebs or ethanol at 1 µl ml⁻¹ in either duodenal muscle. However, at concentrations of 2.3 and 3.3 µl ml⁻¹, ethanol reduced the ACh-induced contractions in longitudinal (25 and 29%) and circular smooth muscle (30 and 30%) respectively (Fig. 1).

**Effect of K⁺ channel blockers on ethanol-induced inhibition in ACh responses**

Apamin (100 nM), glibenclamide (100 nM) and TEA (5 mM) did not affect ethanol-induced inhibition (2.3 µl ml⁻¹) of ACh (0.1 mM) responses in longitudinal or circular smooth muscle. ChTX (10 nM) did not produce any effect ‘per se’ on ACh-induced contractions in longitudinal (88.2 ± 4.8, N = 8) and circular smooth muscle (94.1 ± 10.2, N = 8) with respect
ACh-induced contractions in both muscles (Fig. 2).

Contrast, IbTX (10 nM) increased ethanol-induced inhibition on ACh-induced contractions in two muscles. In control, ChTX (10 nM) antagonized ethanol-induced inhibition on ACh-induced contractions in both muscles (Fig. 2).

Fig. 1. Recording of effect of ethanol (EtOH, 2.3 µl ml⁻¹) for 90 min of incubation on spontaneous contractions and on ACh-induced contractions in longitudinal and circular smooth muscle of rabbit duodenum. Arrowheads indicate addition of acetylcholine (ACh, 0.1 mM), ethanol (EtOH, 2.3 µl ml⁻¹), or washing (W).

Table 1. Effect of ethanol (1, 2.3 and 3.3 µl ml⁻¹) after 90 min of incubation on amplitude and frequency of spontaneous contractions of longitudinal and circular smooth muscle from rabbit duodenum. Values are the mean ± SEM (% of controls) and numbers of segments are in brackets

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<th>Longitudinal Muscle</th>
<th>Circular Muscle</th>
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<td></td>
<td>Amplitude Frequency</td>
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<tr>
<td>Krebs</td>
<td>93.0 ± 5.8 (8) 96.6 ± 3.0 (8)</td>
<td>95.6 ± 4.6 (7) 97.1 ± 4.6 (12)</td>
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<tr>
<td>EtOH 1.0 µl/ml</td>
<td>80.6 ± 5.1 (14) 93.6 ± 1.3 (16)</td>
<td>83.4 ± 6.6 (16) 93.8 ± 3.1 (12)</td>
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<tr>
<td>EtOH 2.3 µl/ml</td>
<td>65.4 ± 6.2 (10)** 92.3 ± 4.6 (11)</td>
<td>73.7 ± 6.7 (8)* 90.9 ± 7.0 (8)</td>
</tr>
<tr>
<td>EtOH 3.3 µl/ml</td>
<td>63.1 ± 5.1 (15)** 103.0 ± 1.9 (8)</td>
<td>75.2 ± 5.5 (12)** 98.3 ± 3.0 (11)</td>
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* P < 0.05, ** P < 0.01, *** P < 0.001 vs. Krebs.

Table 2. Effect of ethanol (EtOH, 2.3 µl ml⁻¹) after 90 min of incubation and action of apamin (AP, 100 nM), charybdotoxin (ChTX, 10 nM), berotoxin (ibTX, 10 nM), glibenclamide (GB, 100 nM) or tetraethylammonium (TEA, 5 mM) added 15 min before ethanol on amplitude and frequency of spontaneous contractions of longitudinal and circular smooth muscle from rabbit duodenum. Values are the mean ± SEM (% of controls) and numbers of segments are in brackets

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<td>AP + EtOH</td>
<td>56.2 ± 5.6 (8)** 110.7 ± 2.8 (8)**</td>
<td>52.8 ± 7.9 (8)** 107.8 ± 5.5 (8)</td>
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<tr>
<td>ChTX + EtOH</td>
<td>105.9 ± 6.4 (12)** 105.2 ± 7.7 (9)</td>
<td>111.3 ± 7.0 (8)** 106.1 ± 8.2 (8)</td>
</tr>
<tr>
<td>IbTX + EtOH</td>
<td>48.7 ± 5.5 (11)** 104.4 ± 2.4 (9)**</td>
<td>78.5 ± 6.6 (8)* 95.3 ± 6.6 (10)</td>
</tr>
<tr>
<td>GB + EtOH</td>
<td>60.4 ± 2.9 (10)** 105.1 ± 2.6 (8)**</td>
<td>64.6 ± 8.3 (10) 98.0 ± 3.3 (9)</td>
</tr>
<tr>
<td>TEA + EtOH</td>
<td>59.1 ± 11.7 (9)** 100.5 ± 5.9 (12)</td>
<td>66.3 ± 11.0 (8) 91.1 ± 12.6 (9)</td>
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* P < 0.05, ** P < 0.01, *** P < 0.001 vs. Krebs and † P < 0.05, †† P < 0.01, ††† P < 0.001 vs. EtOH.

DISCUSSION

The data from these experiments demonstrate that incubation with ethanol (for 90 min) reduced the amplitude of
spontaneous contractions and ACh-induced contractions in longitudinal and circular smooth muscle of rabbit duodenum at two highest concentrations (2.3 and 3.3 µl ml⁻¹). However, the lowest concentration (1 µl ml⁻¹) did not modify these parameters (Table 1). The effect of ethanol was concentration-dependent on ACh-induced contractions and the amplitude of spontaneous contractions in longitudinal muscle. Similarly, ethanol and some alcoholic beverages have been reported to inhibit oesophageal contractility (Keshavarzian et al., 1994) and the amplitude and frequency of spontaneous gastric contractions in cats (Sim et al., 2002). In addition, ethanol decreased spontaneous contractile amplitude in canine jejunal circular smooth muscle (Lu et al., 1997). In humans, the acute and chronic effects of alcohol and alcoholic beverages have an effect on gastric emptying and the motility of the small intestine and colon (Franke et al., 2004). However, ethanol (20–500 mM) produced contractile action in guinea pig gastric longitudinal and circular smooth muscle preparations (Zheng et al., 1997).

Our results, showing that ethanol inhibits the ACh-induced contractions in rabbit duodenum, are consistent with some studies in which ethanol reduced the contractions produced by ACh as well as those produced by periartrial nerve stimulation in isolated rat ileum (Wals et al., 1987). The contractions evoked by ACh, CCK and substance P were also decreased by ethanol in canine jejunal circular smooth muscle (Lu et al., 1997). In alcoholic rats, the sensitivity of the antrum to acetylcholine was four times less than that of control specimens (Palasciano et al., 1995). EtOH strongly inhibited the basal, carbachol, erythromycin, and CCK-stimulated rabbit sphincter of Oddi motility (Sári et al., 2004).

Ethanol’s mechanism of action in smooth muscle of gut is still unclear. We know that it may depolarize the cell membrane and cause release of intracellular calcium, which is, in part, responsible for the contraction produced by ethanol in the rat ileum (Wals et al., 1987). The availability of extracellular calcium partially reversed the reduction of jejunal circular smooth muscle contractility evoked by ethanol (Lu et al., 1997). Furthermore, the inhibition of gastric emptying induced by ethanol involved CCK receptors (Izbeki et al., 2004) and capsaicin-sensitive afferent nerves (Izbeki et al., 2002).

In our work, apamin, iberiotoxin and glibenclamide were not able to revert ethanol-induced dismotility in rabbit duodenum, suggesting that SKCa, BKCa and KATP are not involved. In contrast, charybdotoxin, an IKCa and BKCa blocker, completely cancelled the ethanol-evoked decrease in the amplitude of spontaneous contractions and the ACh-induced response. These results lead us to suggest that ethanol may produce its effects in our model by opening intermediate-conductance Ca²⁺-activated K⁺ channels. Apamin is an SKCa channel blocker. Iberiotoxin, charybdotoxin and TEA are BKCa channel blockers, however ChTX also blocks other types of potassium channels, such as IKCa and SKCa (Farrugia, 1999; Ghatta et al., 2006). In other experimental models, BKCa channels control depolarization and contraction in vascular smooth muscle, and have been involved in the ethanol effect. In this way, ethanol inhibition of the activity of BKCa channels in aortic myocytes may contribute to the direct contraction of aortic smooth muscle produced by acute alcohol exposure (Dopico, 2003). Alcohol consumption in clinically relevant amounts may alter the contribution of maxi-K channels to the regulation of bovine arterial tone (Walters et al., 2000). Our results agree, in part, with these reports.

Our findings suggest that Ca²⁺-activated K⁺ channels are involved in motility disorders evoked by ethanol on spontaneous contractions and ACh-induced contractions in rabbit duodenum muscle.

**Fig. 2.** Effects of ethanol (EtOH, 2.3 µl ml⁻¹) after 90 min of incubation and action of apamin (AP, 100 nM), charybdotoxin (ChTX, 10 nM), iberiotoxin (IBTX, 10 nM), glibenclamide (GB, 100 nM) or tetraetylammonium (TEA, 5 mM) added 15 min before ethanol on ACh-induced contractions in longitudinal and circular smooth muscle from rabbit duodenum. Columns are mean percentage values of integrated mechanical activity in ACh-induced contractions (100% of control) and vertical bars indicate SE. *P < 0.05, **P < 0.01, ***P < 0.001 versus Krebs.
Acknowledgements — This work was funded by the Spanish Ministry of Science and Technology (DGI AGL2003-03291 and ERF). A grant to DS Fageda was provided by Grupo de Investigación Consolidado de Gobierno de Aragón B61/2006 (Spain).

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