Effects of ethanol and other aliphatic alcohols on NO-mediated relaxations in rat anococcygeus muscles and gastric fundus strips

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1 In anococcygeus muscles, ethanol (20–500 mM) slightly increased the tone and inhibited relaxations elicited by nitrenergic nerve stimulation (0.5–5 Hz) in a concentration-dependent manner.

2 Other aliphatic alcohols decreased the tone but had inhibitory effects similar to ethanol on stimulation-induced relaxations, the EC₅₀ (mM) values being: methanol 280, ethanol 80, propan-1-ol 20, propan-2-ol 55, propan-1,2-diol 135, butan-1-ol 120, butan-2-ol 15 and pentan-1-ol 3.

3 Relaxations induced by sodium nitroprusside (SNP, 10 nM) were inhibited by ethanol (20–500 mM) in a concentration-dependent manner and by propan-2-ol (100 mM). Relaxations induced by NO (1 µM) were inhibited by high concentrations of ethanol (200–300 mM) and by propan-2-ol (100 mM).

4 In gastric fundus strips, ethanol (60–200 mM) did not affect the resting tone but inhibited NO-mediated relaxations elicited by low frequency (1 Hz) field stimulation and reduced the initial relaxation by high frequency field stimulation (10 Hz) and by SNP (50 nM). The relaxant action of isoprenaline (10 nM) was not reduced although it was slightly slower in onset. Other aliphatic alcohols tested decreased the tone and inhibited relaxations elicited by field stimulation.

5 Acetaldehyde (1–10 mM) inhibited relaxations elicited by field stimulation and SNP in both the rat anococcygeus muscles and gastric fundus strips. The tone of gastric fundus strips was decreased by acetaldehyde but it was transiently increased in anococcygeus muscles.

6 The dehydrogenase inhibitors, 4-methylpyrazole (100 µM) and disulfiram (100 µM) did not significantly affect the inhibition by ethanol of nitrenergic nerve stimulation-induced relaxations in anococcygeus muscles, which suggests that ethanol acts directly.

7 The inhibition of NO-mediated relaxations by alcohols is attributed to sequestration of NO to form nitroso-alcohols.

Keywords: Acetaldehyde; alcohols (aliphatic); anococcygeus muscle (rat); ethanol; gastric fundus strips (rat); nitric oxide; nitrenergic transmission; sodium nitroprusside

Introduction

It has been previously reported that relaxations elicited by stimulation of non-adrenergic, non-cholinergic (NANC) nerves in the rat and rabbit anococcygeus and the bovine retractor penis muscles are inhibited by ethanol (Gillespie & McKnight, 1978; Gillespie *et al.*, 1982). However, the mechanism of this inhibitory effect of ethanol has not been elucidated. More recently, it has been shown that nitrenergic transmission is responsible for the NANC relaxations in the above-mentioned tissues as well as in a number of other tissues, such as the rat gastric fundus, the relaxations being mediated by nitric oxide (NO) or a NO-like substance synthesized from L-arginine (for reviews, see Rand, 1992a,b; Sanders & Ward, 1992; Sneddon & Graham, 1992). Substances that inhibit NO production, such as N^G-nitro-L-arginine methyl ester (L-NAME), or reduce the availability of NO, such as haemoglobin, or inhibit guanosine 3':5'-cyclic monophosphate (cyclic GMP) generation by guanylate cyclase, such as methylene blue, inhibit nitrenergic transmission (for reviews, see Rand, 1992a,b). Hence it is possible that ethanol may interfere with NO-mediated mechanisms in such a way that nitrenergic transmission is inhibited. In a preliminary study, we confirmed the findings of Gillespie and his colleagues in the rat anococcygeus muscle (Li & Rand, 1991). Additionally, we showed that nitrenergic relaxations of the rat gastric fundus strips as well as relaxations to the NO donor, sodium nitroprusside (SNP) in both anococcygeus muscles and gastric fundus strips were inhibited by ethanol, suggest-

ing a common mechanism may be involved. Furthermore, acetaldehyde was found to have a similar but more potent effect than ethanol in inhibiting nitrenergic relaxations in the rat anococcygeus muscles (Li & Rand, 1991). Therefore, we have now further tested the possibility that effects of ethanol may be mediated by acetaldehyde by using inhibitors of alcohol and aldehyde dehydrogenase.

One possible explanation for the inhibition by ethanol of NO-mediated responses is that the alcoholic hydroxyl group is nitrosylated by NO originating either from nitrenergic nerves or NO-releasing compounds such as SNP, resulting in the formation of ethyl nitrite (CH₃-CH₂-O-N=O), and this sequestering of NO reduces its availability for activating guanylate cyclase. If this is the case, other aliphatic alcohols would be expected to have similar activity. In this study we investigated the effects of eight aliphatic alcohols, differing in the numbers of carbon atoms and the position of the hydroxyl group, on the relaxations elicited by nitrenergic stimulation in the rat anococcygeus muscle and in some cases compared their effects on relaxations elicited by exogenously applied nitric oxide (NO) and sodium nitroprusside (SNP). Some of these findings have been communicated to a meeting of the Australian Physiological and Pharmacological Society (Rand *et al.*, 1992).

Methods

Male Sprague-Dawley rats (250–400 g) were killed by decapitation and the anococcygeus muscles and stomach were removed.

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Anococcygeus muscles were set up as described previously (Gillespie 1972; Li & Rand, 1989). The tone was raised and nitrergic relaxations in response to field stimulation were revealed by guanethidine ($10\text{--}30\text{ }\mu\text{M}$) or the α_2 -adrenoceptor agonist, UK14304 ($1\text{ }\mu\text{M}$); there were no apparent differences between the findings with either agent.

A longitudinal strip of the gastric fundus was prepared and set up for recording field stimulation or drug-induced relaxations as described by Li & Rand (1990).

The composition of the physiological salt solution (PSS) used for both tissues was as follows (mM): NaCl 118, KCl 4.7, NaHCO_3 25, MgSO_4 0.45, KH_2PO_4 1.03, CaCl_2 2.5, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14.

The effects of alcohols were studied after initial control responses to nerve stimulation or agonists had been obtained and the alcohol was added to the organ bath in cumulatively increasing concentrations at intervals of 3–5 min. Only one alcohol was used on each preparation. In the experiments with dehydrogenase inhibitors in the anococcygeus muscle, the inhibitor was added 30 min before the tone was raised. Parallel experiments, without addition of alcohols, were elicited in the contralateral muscle from the same donor rat to serve as a time control.

The drugs used were acetaldehyde (BDH), guanethidine sulphate (Ciba), 5-hydroxytryptamine creatinine sulphate (serotonin, Sigma), isoprenaline hydrochloride (Winthrop, Australia), L-phenylephrine hydrochloride (Sigma), 4-methylpyrazole hydrochloride (Sigma), disulfiram (Sigma), nitric oxide (compressed gas, CIG, Melbourne), N^G -nitro-L-arginine methyl ester (L-NAME, Wellcome Research Laboratory, UK), sodium nitroprusside (SNP, Sigma) and UK14304 (5-bromo-6-[2-imidazoline-2-Y1-amino]-quinoxaline; Pfizer). The following alcohols were purchased from BDH: methanol, ethanol, propan-1-ol, propan-2-ol, propan-1,2-diol, butan-1-ol, butan-2-ol and pentan-1-ol. Saturated aqueous solutions of NO were prepared from NO gas as previously described (Rajanyagam *et al.*, 1993).

Differences between means were compared by Student's *t* test, and values of $P < 0.05$ were taken to indicate statistical significance.

Results

Effects of ethanol and other alcohols on field stimulation-induced relaxations and resting tension of rat anococcygeus muscles

Ethanol (20–500 mM) produced a concentration-dependent inhibition of relaxations elicited by field stimulation at various frequencies (0.5–5 Hz) of partially contracted anococcygeus muscle, as shown for 60 and 200 mM ethanol in Figure 1. The reductions were more marked at the lower than at the higher frequencies of stimulation. The resting tone of the anococcygeus muscle was slightly but regularly increased (Figure 1); however, the increase in tone in the prolonged presence of a high concentration of ethanol was not sustained.

The effects of the five primary aliphatic alcohols from methanol to pentan-1-ol, two secondary alcohols (propan-2-ol and butan-2-ol) and the di-alcohol propan-1,2-diol were tested. All of these alcohols produced concentration-dependent reductions of nitrergic nerve stimulation-induced (1 Hz, 10 s) relaxations as shown in Figure 2a,b. The mean concentrations that reduced these relaxations by 50% (EC_{50} values) are shown in Table 1. All of the alcohols except ethanol decreased tone, and this created difficulties for the precise assessment of the ED_{50} values; hence, only approximate means are given. The relationship between the reduction in tone and the inhibition of nitrergic relaxation by the alcohols is shown in Figure 3.

Figure 1 Records of effects of ethanol (upper tracings) and acetaldehyde (lower tracing) on relaxations of anococcygeus muscles elicited by field stimulation (0.5–5 Hz for 10 s, at (O) in the upper tracings and 5 Hz for 10 s at (●) in the lower tracings). Middle tracings show the effect of ethanol on relaxations of an anococcygeus muscle elicited by sodium nitroprusside (SNP, 10 nM). In these records, the tone of the muscles was raised by guanethidine. Similar results were obtained on each type of response in at least 5 separate experiments.

Effects of ethanol and propan-2-ol on SNP- and NO-induced relaxations of anococcygeus muscles

Relaxations elicited by SNP (10 nM) were inhibited by ethanol (60–200 mM) in a concentration-dependent manner (Figures 1 and 4). Relaxations elicited by NO ($1\text{ }\mu\text{M}$) were not significantly inhibited by 100 mM ethanol but there was significant inhibition with 200–300 mM ethanol (Figure 4). Propan-2-ol (100 mM) significantly inhibited relaxations induced by both SNP (10 nM) and NO ($1\text{ }\mu\text{M}$) (results not shown).

Table 1 The EC_{50} (mM) of the alcohols tested in inhibiting nitrergic relaxations of the anococcygeus

Alcohol	EC_{50} (mM)	Alcohol	EC_{50} (mM)
Methanol	280	Pentan-1-ol	3
Ethanol	80	Butan-2-ol	15
Propan-1-ol	20	Propan-1-ol	20
Propan-2-ol	55	Propan-2-ol	55
Propan-1,2-diol	135	Ethanol	80
Butan-1-ol	120	Butan-1-ol	120
Butan-2-ol	15	Propan-1,2-diol	135
Pentan-1-ol	3	Methanol	280

The first two columns are in order of size and complexity of the alcohols; the second two columns are in order of potency.

Figure 2 Effects of aliphatic alcohols on field stimulation-induced (1 Hz, 10 s) relaxations of anococcygeus muscles, expressed as percentage reduction of the relaxations (a and b). (a) (O) methanol; (●) ethanol; (▲) propan-1-ol; (△) propan-2-ol. (b) (□) Propan-1,2-diol; (■) butan-1-ol; (◇) butan-2-ol; (◆) pentan-1-ol. Symbols represent means ($n = 3-7$) with standard errors of means, which in some cases are less than the size of the symbol indicating the mean.

Figure 3 The relationship between the mean changes in tone and mean inhibitions of nitrgic relaxations by aliphatic alcohols: (O) methanol; (●) ethanol; (▲) propan-1-ol; (△) propan-2-ol; (□) propan-1,2-diol; (■) butan-1-ol; (◇) butan-2-ol; (◆) pentan-1-ol.

Effects of ethanol and other alcohols on NO-mediated relaxations in gastric fundus strips

In gastric fundus strips, as in anococcygeus muscles, relaxations elicited by field stimulation were reduced in a concentration-dependent manner by ethanol (60–200 mM), which did not greatly affect the resting tone (Figure 5); the effect of ethanol was considerably greater on the relaxation elicited by 1 Hz for 60 s than that elicited by 5 Hz for 10 s. Ethanol also inhibited the initial rapid phase of the relaxation elicited by high frequency stimulation (10 Hz for 60 s), as shown in Figure 5 with 200 mM of ethanol. The relaxant action of SNP was almost abolished by 200 mM ethanol, as was the

Figure 4 Effects of ethanol on relaxations elicited by NO (open columns) and sodium nitroprusside (SNP) (solid columns) in rat anococcygeus muscles, expressed as percentage of the tone of the muscles raised by guanethidine. Columns represent means ($n = 3-7$) with standard errors of means. *Indicates significantly different ($P < 0.05$) from control (no ethanol).

Figure 5 Records of effects of ethanol on relaxations of rat gastric fundus strips, in which the tone was raised by 5-hydroxytryptamine (5-HT, $10 \mu\text{M}$), elicited by field stimulation at 1 Hz for 60 s (indicated by horizontal lines), 5 Hz for 10 s (indicated by O) and 10 Hz for 60 s (indicated by horizontal lines), sodium nitroprusside (SNP, 50 nM) and isoprenaline (Iso, 10 nM). Periods of exposure to drugs are indicated by horizontal lines. Similar results were obtained on each type of response in at least 4 separate experiments.

stimulation (1 Hz)-induced relaxation, but the relaxant action of isoprenaline was not reduced although it was slightly slower in onset (Figures 5 and 6).

The other alcohols tested on gastric fundus strips (propan-2-ol and butan-1-ol) produced concentration-dependent inhibitions of relaxations to field stimulation but also greatly reduced the tone of the muscle (results not shown).

Figure 6 Effects of ethanol on relaxation elicited by field stimulation (Stim, 5 Hz, 10 s), sodium nitroprusside (SNP, 50 nM) and isoprenaline (Iso, 10 nM) in rat gastric fundus, expressed as percentage of the initial relaxations. Columns represent means ($n = 4-7$) with standard errors of means. *Indicates significantly different ($P < 0.05$) from control (no ethanol).

Effects of acetaldehyde on NO-mediated relaxations in anococcygeus muscles and gastric fundus strips

In anococcygeus muscles, acetaldehyde (1–10 mM) had concentration-dependent effects in increasing the tone and inhibiting nitrgic nerve stimulation-induced relaxations as shown in Figure 1. Thus, acetaldehyde was about 100 times more potent than ethanol in both actions.

In the gastric fundus strip, acetaldehyde (1–10 mM) also reduced nitrgic nerve stimulation-induced relaxations and was again more potent than ethanol, but reduced the resting tone (results not shown).

Relaxations of anococcygeus muscles and gastric fundus strips elicited by SNP (10 nM) were inhibited by 3–10 mM acetaldehyde to about the same extent as by 200 mM ethanol (results not shown).

Effects of 4-methylpyrazole and disulfiram on the inhibition of nitrgic relaxation by ethanol in the anococcygeus muscle

Neither 4-methylpyrazole (100 μ M) nor disulfiram (100 μ M) had any significant effect on the inhibition by ethanol (20–200 mM) of relaxations elicited by nitrgic nerve stimulation or SNP (10 nM) (results not shown).

Discussion

The present study confirmed and extended the previous observations that ethanol inhibited NANC-mediated relaxations in the rat anococcygeus muscle and related tissues (Gillespie & McKnight, 1978; Gillespie *et al.*, 1982; Li & Rand, 1991). In addition we have shown that acetaldehyde and other aliphatic alcohols have effects similar to ethanol in both the rat anococcygeus muscle and the gastric fundus.

Ethanol slightly increased the tone of anococcygeus muscle, as previously reported by Gillespie *et al.* (1982), who also found that ethanol potentiated the contractile responses to noradrenergic nerve stimulation. Both these effects are produced by other agents that inhibit nitrgic nerve-stimulation-induced relaxations, such as NO synthase inhibitors, haemoglobin and methylene blue (Rand, 1992a), and are attributable to removal of a NO-mediated relaxant influence. However, ethanol also reduced the contractile action of noradrenaline (Gillespie *et al.*, 1982).

In gastric fundus strips, ethanol selectively inhibited NO-mediated relaxations elicited by field stimulation at a low frequency (1 Hz) and reduced the initial rapid relaxation at high frequency (10 Hz), and it has been established that this

relaxation is mediated by a nitrgic mechanism (Li & Rand, 1990). The inhibition of SNP- but not isoprenaline-induced relaxations by ethanol is in accord with the concept of a selective inhibition by ethanol of a NO-mediated process.

It has been reported that ethanol inhibited the NO-mediated, endothelium-dependent relaxant actions of acetylcholine and ATP but not the NO-independent relaxant action of papaverine in the rat mesenteric artery (Criscione *et al.*, 1989). We have also found that both acetylcholine- and SNP-induced relaxations of rat aortic rings were inhibited by ethanol and propan-2-ol (Li & Rand, unpublished observations). However, our finding that ethanol inhibited the relaxant action of SNP in the rat aorta differs from that reported by Hatake *et al.* (1989), who found that ethanol inhibited only acetylcholine- but not SNP-induced relaxations. The reason for this discrepancy is not clear.

Acetaldehyde had effects similar to ethanol in the rat anococcygeus and gastric fundus. The possibility of production of acetaldehyde from ethanol in isolated extrahepatic tissues has been discussed by Altura *et al.* (1978). Therefore, we tested the possibility that the inhibitory effect of ethanol was mediated by acetaldehyde by using high concentrations of 4-methylpyrazole and disulfiram which are selective inhibitors of alcohol and aldehyde dehydrogenases, respectively. However, since neither 4-methylpyrazole nor disulfiram significantly affect the actions of ethanol in the rat anococcygeus muscle, it seems unlikely that the effects of ethanol were mediated by acetaldehyde. Thus, acetaldehyde may, in its own right, inhibit the nitrgic responses by either the same or a different mechanism from ethanol. More experiments will be necessary before any conclusion can be drawn.

Gillespie *et al.* (1982) suggested that the inhibition by ethanol of NANC-mediated relaxations in anococcygeus muscles and the bovine retractor penis were most probably due to an alteration in the level of intracellular calcium in the smooth muscle cells. At that time, the mechanism involved in mediating the NANC relaxations had not been elucidated, but more recently it has been shown that the NANC relaxant transmitter in these tissues is NO, or a NO-like substance (for reviews, see: Rand, 1992a,b; Sanders & Ward, 1992; Sneddon & Graham, 1992). In this context, an alternative explanation for the effect of ethanol is that it may inhibit one or more stages in the nitrgic transmitter mechanism.

Since the relaxations of anococcygeus muscle to exogenously applied NO and the NO-donor SNP were also inhibited, it is unlikely that the effect of ethanol was due to inhibition of transmitter release from nitrgic nerves. The findings could be explained either by inhibition of the guanylate cyclase/cyclic GMP mechanism mediating the relaxation of the smooth muscle, or by an interaction of ethanol with the released NANC transmitter, NO or a NO-related compound, resulting in its inactivation. However, SNP-induced relaxations of the bovine retractor penis muscle were not affected by ethanol (Gillespie *et al.*, 1982), although it reduced the response to the inhibitory factor (IF) isolated from the bovine retractor penis muscle (Gillespie *et al.*, 1982), which has recently been identified as a NO-donating nitrosothiol (Kerr *et al.*, 1992).

One of the possible interactions of ethanol with NO is formation of the nitroso compound ethyl nitrite ($\text{CH}_3\text{-CH}_2\text{-O-N=O}$). If this is the case, other alcohols may be expected to have a similar effect. The finding that all the other aliphatic alcohols tested inhibited nitrgic relaxations is in accord with this concept. According to Taylor & Baker (1945), the reaction of NO with alcohols to form nitroso-alcohols ($\text{NO} + \text{R-OH} \rightarrow \text{R-O-N=O}$) proceeds readily. Certain nitroso-alcohols (e.g. butyl and amyl nitrite, or nitrosobutanol and nitrosopentanol, respectively, as they may also be termed) are well known as nitrovasodilators, which donate NO to guanylate cyclase and thereby relax smooth muscle. We suggest that the reaction of NO with an alcohol is reversible, and NO is sequestered when the alcohol is in excess, but NO is released when the nitroso-alcohol is in

excess. The differences in potency of the alcohols may reflect the equilibrium position of their reactions with NO under the prevailing conditions. In general, an increase in chain length of an alcohol resulted in an increase in its ability to inhibit nitrenergic relaxations; however, as other factors are also involved (such as the reduction in tone), the potency for each alcohol is only approximate. Evidence for an interaction between NO and ethanol (as we propose), or a competition between NO and ethanol for a binding site, was provided by Nelson (1987), who observed that the pH-dependent changes in the electron paramagnetic resonance spectrum of the NO complex with the non-haem iron containing enzyme soybean lipoxygenase were abolished by 100 mM ethanol (Nelson, 1987).

The amount of endogenously formed NO in the exhaled air of anaesthetized rabbits was decreased in a dose-dependent manner by infusions of 3, 10 and 30 mmol kg⁻¹ ethanol (Persson & Gustafsson, 1992). The ED₅₀ for inhibition of exhaled NO was 23 mmol kg⁻¹ and, given that the volume of distribution of ethanol in rabbits is about 0.65 l kg⁻¹, the ethanol concentration in body water would be about 35 mM ethanol, which is not too divergent from our estimate of an EC₅₀ of about 80 mM for inhibition of nitrenergic relaxations of anococcygeus muscles. Persson & Gustafsson (1992) attributed the effect of ethanol to inhibition of NO synthesis; however, the finding is compatible with our suggestion that the NO is sequestered by formation of ethyl nitrite.

It was noted that there were some differences in the sensitivity to inhibition of NO-mediated relaxations by ethanol in different tissues. For example, ethanol inhibited nitrenergic relaxations in the rat anococcygeus muscle and gastric fundus strip at a much lower concentration than that needed to inhibit endothelium-dependent relaxations in the aorta (unpublished observation). It is not clear whether the difference is due to a difference in the fate of the NO after it is released from its source (nerve terminals or endothelial cells) or to the possible different nature of nerve-derived and endothelium-derived NO (Rand & Li, 1993). Furthermore, ethanol was found to be less effective against NO-induced relaxation than that induced by SNP or nitrenergic nerve stimulation. Again, it

is not clear whether this reflects the nature of the equilibrium of the proposed reaction between ethanol and NO or whether ethanol has an additional effect on NO synthesis. In addition, effects other than interaction with NO may also be involved in the actions produced by alcohols, especially at the relatively high concentrations used, since the tone of the muscle was decreased by all the alcohols except ethanol. It is possible that this reduction in tone is due to disordering of the cell membrane by the alcohols, causing a reduction of the intracellular calcium concentration in the smooth muscle cells, as suggested by Gillespie *et al.* (1982).

The demonstration that ethanol as well as other alcohols impair nitrenergic transmission may be of some significance in understanding the possible mechanism of functional changes in a variety of physiological systems that are observed in alcohol abuse. For example, in the central nervous system, the activation of neuronal guanylate cyclase by excitatory amino acid agonists, such as glutamate and NMDA, is mediated by NO (Bredt & Snyder, 1989). Ethanol reduces the levels of cyclic GMP in the brain, particularly in the cerebellum (for references, see Hoffman *et al.*, 1989a), and the production of cyclic GMP stimulated by agonists in the cerebellar granule cells (Hoffman *et al.*, 1989a,b) and other cell lines (Stenstrom *et al.*, 1986) was inhibited by ethanol. Ethanol also reduced the neurotoxicity of glutamate and NMDA in cultures of rat cerebral cortical cells (Lustig *et al.*, 1992), and this effect is thought to be mediated by NO. However, as was pointed by Hoffman *et al.* (1989b) and Stenstrom *et al.* (1986), the inhibition of agonist-stimulated production of cyclic GMP by ethanol may not be due to inhibition of guanylate cyclase. Ethanol inhibited SNP-stimulated cyclic GMP production in cultured neuroblastoma cells (Stenstrom *et al.*, 1986); this effect could be due to inhibition of guanylate cyclase, or to sequestration of the NO released from SNP.

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