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ETHANOL INHIBITS DUAL RECEPTOR STIMULATION OF PINEAL cAMP AND cGMP BY VASOACTIVE INTESTINAL PEPTIDE AND PHENYLEPHRINE

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SUMMARY: Concurrent activation of vasoactive intestinal peptide and α_1-adrenergic receptor resulted in greater than 20-fold increases in pineal cAMP and cGMP accumulation. We now find that an intoxicating level of ethanol (0.2%, 34 mM) inhibits >50% the large increases in pineal cAMP and cGMP produced by concurrent treatment with vasoactive intestinal peptide and phenylephrine. The potency of the various alcohols tested was directly related to their chain length. This inhibition appears to be specific since a five-fold higher concentration of ethanol does not inhibit the stimulation of cAMP and cGMP accumulation produced by concurrent treatment with isoproterenol and phenylephrine. Accordingly, it seems that one mechanism of action of ethanol on neural function may be its ability to selectively inhibit ethanol-sensitive integrative mechanisms which regulate cyclic nucleotides.

An exciting development in our understanding of transmembrane regulation of cyclic nucleotides in neural tissue is that two seemingly independent receptor mechanisms can interact to produce much larger changes in cyclic nucleotide levels than would be predicted from the effects produced by activation of either receptor alone (1-7). For example, activation of α_1-adrenergic receptors has little apparent effect by itself on pinealocyte cAMP and cGMP accumulation, but potentiates by 10- to 30-fold the effects of β-adrenergic or vasoactive intestinal peptide (VIP) stimulation (6-7)[2]. This explains the 30- to 100-fold increases in both cyclic nucleotides which occur when pinealocytes are treated with isoproterenol and phenylephrine, or VIP and phenylephrine, or norepinephrine, which activates both α_1- and β-adrenoceptors. These dual receptor regulatory systems are of potential

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[2]We have recently found that combined VIP and phenylephrine treatment stimulated cAMP 20-fold and cGMP 60-100 fold (Chik, C.L., Ho, A.K. and Klein, D.C., submitted).

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importance in neural signal processing because they could function as highly selective neurochemical gating mechanisms, which integrate multiple synaptic input.

The more complex nature of these integrative systems, as compared to the one receptor-one cyclic nucleotide systems which have been well studied, carries with it the potential for drugs to act by inhibiting the mechanism involved in the interaction, rather than by inhibiting either system. We now report the first example of this: an intoxicating level (8) of ethanol (0.2%, 34 mM) blocks the stimulation of cAMP and cGMP accumulation by the concurrent activation of VIP receptors and α_1-adrenoceptors. In contrast, a 5-fold higher level of ethanol does not inhibit the effects of isoproterenol, VIP, or norepinephrine, or the combination of isoproterenol and phenylephrine.

METHODS

Synthetic porcine VIP was purchased from Peninsula Laboratories (San Carlos, Ca) and isoproterenol, phenylephrine and norepinephrine were obtained from Sigma Chemical Corp. (St. Louis, MO). All other drugs and chemicals were from commercial sources and were of the purest grade available. Antibodies for the radioimmunoassays of cAMP and cGMP were gifts from Dr. K. Catt (NICHD, NIH, Bethesda, MD).

Pinealocytes were prepared from rat (Sprague-Dawley, 200 gm female) pineal glands by enzymatic and physical dispersion as previously described (9). Cells were maintained in Dulbecco's modified Eagle's Medium containing 10% fetal calf serum under an atmosphere of 95% oxygen and 5% CO_2 at 37°C for 24 hours. Cells were then transferred to individual tubes (10^5 cells/0.5 ml) and treated with drugs of interest for 15 min. At the end of the treatment period, cells were collected by centrifugation (1000 x g, 2 min) and placed on solid CO_2. Cyclic nucleotides were measured by radioimmunoassay in cell pellets (6,10). Protein in cell pellets was measured using a dye binding method with bovine serum albumin as a standard (11).

Data are presented as mean ± SE and statistical analysis was performed using Duncan's multiple range test (12).

RESULTS

The combined treatment with VIP (10^{-6} M) and phenylephrine (10^{-5} M) increased cAMP accumulation 20-fold and increased cGMP accumulation 130-fold (Figure 1; absolute values appear in legend), as previously described (4). Ethanol (0.2%, 34 mM) inhibited the cAMP response by about 50% and was even more effective in inhibiting the cGMP response (Figure 1). In contrast, ethanol (1%, 171 mM) did not inhibit the effects of VIP (10^{-6} M), norepinephrine (10^{-6} M) or the combined treatment with isoproterenol (10^{-8} to 10^{-6} M) and phenylephrine (10^{-5} M) (Table 1, Figure 1). It should be emphasized that the cAMP and cGMP responses to combined isoproterenol (10^{-8} M) and phenylephrine (10^{-5} M) treatment are in the same range as those generated by VIP (10^{-6} M) and phenylephrine (10^{-5} M), yet ethanol

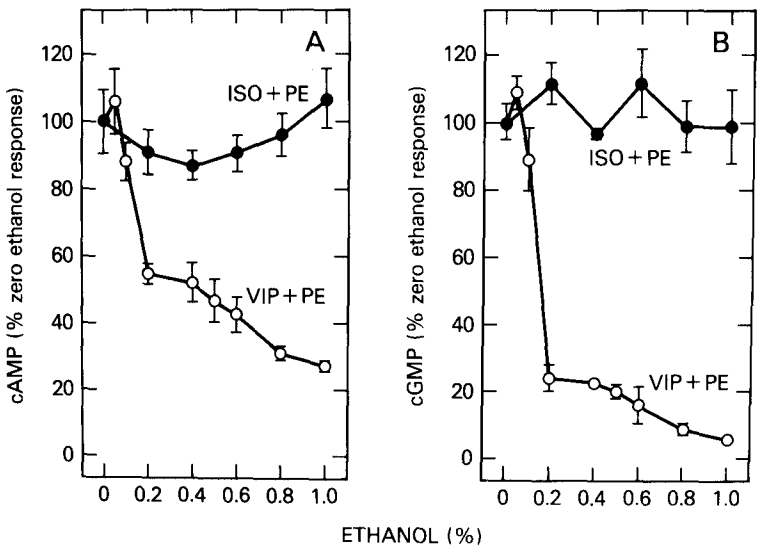


Figure 1. Effect of ethanol on VIP or isoproterenol stimulation of the cAMP and cGMP contents of phenylephrine stimulated rat pinealocytes. Pinealocytes were isolated from rat pineal glands and incubated under control conditions for 24 h. They were then aliquoted (10^5 cells/0.5ml) and incubated for 15 min with VIP (10^{-6} M) + phenylephrine (PE, 10^{-5} M) or isoproterenol (ISO, 10^{-6} M) + PE (10^{-5} M) in the presence or absence of graded concentrations of ethanol. Each point represents the mean ± SE of cAMP and cGMP determinations done in duplicate on three samples of cells. cAMP and cGMP were measured by radioimmunoassay. The absence of an error bar indicates that the SE fell within the area of the symbol. The absolute cAMP values were: untreated controls, 15 ± 1; VIP + PE, 304 ± 32; and, ISO + PE, 2200 ± 203 pmoles/mg protein. The absolute cGMP values were: untreated controls, 1.1 ± 0.3; VIP + PE, 142 ± 8; and, ISO + PE, 941 ± 40 pmoles/mg protein respectively.

inhibited only the latter (Figure 1, Table 1). In contrast to the marked inhibitory effects of ethanol on concurrent VIP and phenylephrine stimulated cyclic nucleotide accumulation, it was observed that ethanol modestly elevated VIP- or isoproterenol-stimulation of cAMP (Table 1), as previously reported (13,14).

One generally accepted action of ethanol is membrane fluidization. This was tested by comparing the inhibitory effects of a series of alcohols of increasing chain length since a direct relationship exits between alcohol chain length and fluidization of membranes (15,16). We found the potency of the alcohols tested was directly related to chain length (Figure 2).

DISCUSSION

Many effects of ethanol on transmembrane signal processing have been reported. This includes modulation of adenylyl cyclase and guanylyl cyclase activities; binding of neurotransmitters, hormones and Ca^{2+}; Ca^{2+} metabolism; and, receptor stimulated cAMP and

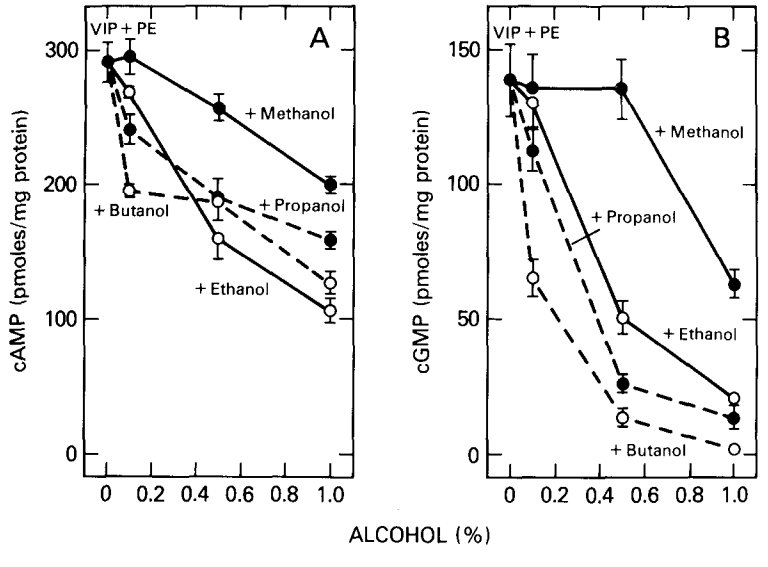
Table 1

Effect of ethanol on cAMP and cGMP contents of VIP- or adrenergic agonist-treated pinealocytes. Pinealocytes were isolated from rat pineal glands and incubated under control conditions for 24 h. Cells were then aliquoted (10^5 cells/0.5 ml) and incubated for 15 min with VIP (10^{-6} M), norepinephrine (10^{-6} M), isoproterenol (10^{-6} M), phenylephrine (10^{-5} M), or combinations of phenylephrine (10^{-5} M) and isoproterenol (10^{-8} or 10^{-7} M), in the presence or absence of ethanol (1%, 171 mM). Each value represents the mean ± SE of cAMP or cGMP determinations done in duplicate on three samples of cells. cAMP and cGMP were measured by radioimmunoassay.

Exp.	Treatment	cAMP (pmoles/mg protein)	cGMP (pmoles/mg protein)
I	Control	11.4 ± 2.0	1.4 ± 0.4
	+ ethanol	12.1 ± 1.5	1.3 ± 0.3
	VIP	55.6 ± 4.4	5.7 ± 1.0
	+ ethanol	76.0 ± 2.0[a]	8.2 ± 0.9
	Norepinephrine	1780 ± 104	1180 ± 35
	+ ethanol	1770 ± 148	963 ± 97
	Isoproterenol	378 ± 36.3	20.3 ± 2.4
	+ ethanol	502 ± 39.3[b]	27.5 ± 4.0
	Phenylephrine	25.3 ± 1.7	4.4 ± 1.3
	+ ethanol	31.7 ± 5.7	4.9 ± 1.1
II	Phenylephrine + isoproterenol (10^{-7} M)	1710 ± 25	599 ± 100
	+ ethanol	1670 ± 90	527 ± 56
	Phenylephrine + isoproterenol (10^{-8} M)	382 ± 50	166 ± 29
	+ ethanol	300 ± 57	135 ± 28

[a]Significantly different from the VIP treated group ($p < 0.05$)

[b]Significantly different from the isoproterenol treated group ($p < 0.05$)



cGMP accumulation (17-21). Most of these effects require 5- to 10-fold higher concentrations of ethanol than those required to produce the inhibitory effects described here, concentrations rarely achieved after consumption of alcohol in humans. In contrast, the concentrations of ethanol in the culture medium required for the effects reported here correspond to plasma concentrations achieved after a modest intake of alcohol (8).

Although the potency of ethanol in the dual VIP and α_1-adrenergic system is impressive, a more intriguing issue is specificity: How does ethanol inhibit the effects of the combined treatment with phenylephrine and VIP, but not the effects of phenylephrine and isoproterenol? It appears that ethanol has a selective effect on the mechanism involved in the interaction of α_1-adrenoceptors and VIP receptors, because it does not inhibit the effects of either VIP, isoproterenol, isoproterenol and phenylephrine, or norepinephrine treatment, all of which appear to act through similar mechanisms to stimulate cAMP and cGMP (22,23). The answer could lie in membrane fluidization, which is one generally accepted action of ethanol (15,16). The direct relationship between potency of inhibition of the various alcohols and their chain length suggests that the mechanism of action of ethanol in this system may indeed be related to membrane fluidization.

The evidence that ethanol acts by increasing membrane fluidity (15,16, Figure 2) leads us to suspect that ethanol might act via physical effects on key membrane bound components of this dual receptor regulatory system. The mechanisms involved in stimulation of pineal cAMP and cGMP are beginning to be understood. The β-adrenergic and VIP stimulation of pineal cAMP and cGMP accumulation appear to involve the increased activity of adenylyl cyclase and probably guanylyl cyclase (22,23). These effects are mediated by GTP binding proteins (24). The effects of α_1-adrenergic stimulation are mediated by the translocation of protein kinase C from the cytoplasm to the membrane (25). The translocation apparently results from an increase in intracellular Ca^{2+} (26) and activation of phospholipase C (27). The basis of α_1-adrenergic potentiation may be to increase the efficiency of β-adrenergic or VIP

Figure 2. Effects of a series of alcohols on stimulation of pineal cAMP and cGMP contents by VIP and phenylephrine. Pinealocytes were isolated from rat pineal glands and incubated under control conditions for 24 h. Cell were then aliquoted (10^5 cells/0.5ml) and incubated for 15 min with VIP (10^{-6} M) + phenylephrine (PE, 10^{-5} M) in the presence or absence of graded concentrations of methanol, ethanol, propanol or butanol. Each point represents the mean $\pm$ SE of cAMP or cGMP determinations done in duplicate on three samples of cells. cAMP and cGMP were measured by radioimmunoassay. The absence of an error bar indicates that SE fell within the area of the symbol.

stimulation of cyclic nucleotide synthesis (28). It is obvious that one difference between the stimulation of cAMP and cGMP by VIP and phenylephrine as compared to that by isoproterenol and phenylephrine is the VIP receptor. Thus, it is likely that an increase in membrane fluidity alters the nature of this dual receptor regulatory mechanism by influencing the interaction between the VIP receptor and a component of the mechanism involved in mediating α_1-adrenergic effects.

Irrespective of the mechanism involved, the knowledge that an intoxicating concentration of ethanol has a relatively selective effect on VIP and phenylephrine regulation of cAMP and cGMP is important by itself. VIP and α_1-adrenergic receptors interact to control cAMP in the cerebral cortex and other brain regions (4,29,30). Blood alcohol levels of up to 0.5% can be attained in humans (31). Perhaps some of the rapid mood altering or disorientating effects of ethanol on human behavior are due to selective effects on dual receptor stimulation of cAMP and cGMP by VIP and α_1-adrenoceptors or by other pairs of transmitters.

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