Biochemical Pharmacology, Vol. 42, No. 8, pp. 1601–1608, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INHIBITORY EFFECTS OF ETHANOL ON THE CALCIUM-DEPENDENT POTENTIATION OF VASOACTIVE INTESTINAL PEPTIDE-STIMULATED cAMP AND cGMP ACCUMULATION IN RAT PINEALOCYTES

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(*Received* 4 *February* 1991; *accepted* 3 *June* 1991)

Abstract—In rat pinealocytes, ethanol has been shown recently to inhibit the α_1-adrenergic potentiation of vasoactive intestinal peptide (VIP)-stimulated cyclic AMP (cAMP) and cyclic GMP (cGMP) responses, with the cGMP response being more sensitive to the inhibition. Two intracellular events known to be of importance to the potentiation mechanism are activation of protein kinase C and elevation of intracellular calcium ($[Ca^{2+}]_i$). In this study, we examined the effects of ethanol on these two intracellular mechanisms with an activator of protein kinase C, and two agents that elevate $[Ca^{2+}]_i$, depolarizing concentrations of K^+ and A23187. Using dispersed pinealocytes, ethanol (up to 175 mM) was found to have no effect on the 4β-phorbol 12-myristate 13-acetate (PMA, an activator of protein kinase C) potentiated VIP-stimulated cAMP response. As for the cGMP response, full potentiation requires both activation of protein kinase C and simultaneous elevation of $[Ca^{2+}]_i$. This could be achieved by stimulating the VIP-treated cells with a combination of PMA and 15 mM K^+. In the presence of ethanol, the amplification effect of PMA and K^+ on the VIP-stimulated cGMP response was inhibited with an IC_{50} value of 125 mM. In contrast, similar concentrations of ethanol had no effect on the corresponding cAMP response. These findings suggest that the potentiation of cAMP response by protein kinase C is not affected by ethanol. When depolarizing concentrations of K^+ were used to potentiate the VIP-stimulated cAMP and cGMP accumulation, ethanol inhibited the K^+ potentiation of VIP-stimulated cAMP and cGMP responses with IC_{50} values of 50 and 30 mM, respectively. The A23187 potentiation of VIP-stimulated cGMP response was also sensitive to the inhibitory effect of ethanol with an IC_{50} value of 120 mM. In comparison, the corresponding IC_{50} value for the cAMP response was >175 mM. Based on our findings, we conclude that ethanol likely inhibits a Ca^{2+}-dependent event(s) that is critical to the α_1-adrenergic-mediated potentiation of VIP-stimulated cAMP and cGMP responses.

The rat pineal gland, a neuroendocrine organ, has provided an excellent system for the studies of neurotransduction processes [1] and ethanol action [2, 3]. The synthesis of the pineal hormone, melatonin, in the rat pineal gland is regulated by the release of norepinephrine (NE) from the sympathetic nerve terminals that innervate the organ [4]. The formation of melatonin depends on the synthesis and activity of the enzyme serotonin *N*-acetyltransferase, a cyclic AMP (cAMP)-dependent enzyme [5]. NE acts via α_1- and β-adrenergic receptors to stimulate cAMP and cyclic GMP (cGMP) accumulation [6, 7].

In addition to NE, the peptide hormone vasoactive intestinal peptide (VIP) also appears to be an important regulator of rat pineal melatonin synthesis. This is based on the findings that VIP receptors are abundant in this tissue [8], that pineal VIP content oscillates on a 24-hr basis [9], and that VIP stimulates cAMP [10, 11] and *N*-acetyltransferase activity [10–12]. Furthermore, in the rat pineal gland, activation of VIP receptors increases both cAMP and cGMP accumulation several-fold [11, 13]. α_1-Adrenergic agonists, which by themselves have little effect on cyclic nucleotide responses, potentiate the VIP-stimulated cAMP accumulation 5-fold and cGMP accumulation 20-fold [11, 13]. The two α_1-adrenergic-mediated intracellular events known to be of importance to the potentiation of VIP-stimulated cyclic nucleotide responses are elevation of intracellular calcium ($[Ca^{2+}]_i$) and activation of protein kinase C [14]. While $[Ca^{2+}]_i$ elevating agents potentiate VIP-stimulated cAMP and cGMP responses equally, and activation of protein kinase C alone is sufficient for the full potentiation of the cAMP response, the potentiation of cGMP by activation of protein kinase C requires a simultaneous elevation of $[Ca^{2+}]_i$ [14].

Recently, the effect of alcohol on the α_1-adrenergic potentiation of VIP-stimulated cAMP and cGMP responses also was investigated. We found that ethanol has a potent inhibitory action on the α_1-adrenergic potentiation of VIP-stimulated cAMP and cGMP responses, with the cGMP response being more susceptible to the inhibitory action of ethanol [2]. In the present study we investigated the possible intracellular mechanisms that may account for the difference in sensitivity between the cAMP and the cGMP responses to ethanol. Specifically, the effects of ethanol treatment on the two post-receptor

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mechanisms known to be involved in the α_1-adrenergic potentiation of VIP-stimulated cAMP and cGMP responses were compared. To evaluate a possible effect of ethanol on the protein kinase C pathway, we examined the effects of ethanol on the 4β-phorbol 12-myristate 13-acetate (PMA) potentiation of cyclic nucleotide responses. For the Ca^{2+}-dependent mechanism, the effects of ethanol on the potentiation of $[Ca^{2+}]_i$-elevating agents on VIP-treated cells were determined.

MATERIALS AND METHODS

Chemicals. The following were purchased: synthetic porcine VIP from Peninsula Laboratories (San Carlos, CA); phenylephrine (PE) and A23187 from the Sigma Chemical Co. (St. Louis, MO); and phorbol esters from Calbiochem (La Jolla, CA). All other chemicals were from commercial sources and were of the purest grades available. Antibodies for the radioimmunoassays of cAMP and cGMP were gifts from Dr. A. Baukal (NICHD, NIH, Bethesda, MD).

Preparation of rat pinealocytes. Pinealocytes were prepared from pineal glands (200 g male Sprague–Dawley rats) by trypsinization and physical treatment as previously described [15]. Cells were then suspended in Dulbecco's Modified Eagle's Medium (containing 10% fetal bovine serum) and incubated for 24 hr in a gas mixture of 95% air/5% CO_2 at 37°. Aliquots of cells (10^5 cells/0.5 mL) were treated with drugs for 15 min. Drugs were dissolved in H_2O or dimethyl sulfoxide with a final concentration of <0.1% per incubation volume. At this concentration, dimethyl sulfoxide has no effect on the cAMP and cGMP responses of pinealocytes to VIP and VIP-mediated responses. After treatment, the cells were collected by centrifugation (2 min, 1000 *g*), the supernatant was aspirated, and the pellet was immediately frozen on solid CO_2.

Cyclic nucleotide assays. The frozen cell pellet was lysed by boiling for 3 min in 5 mM acetic acid (100 μL). The preparation was then centrifuged (12,000 *g*, 10 min), and the supernatant was used for determinations of cAMP and cGMP by radioimmunoassays [16]. When necessary, samples were diluted further with 5 mM acetic acid. Protein in the cell pellet was measured by a dye binding method using bovine serum albumin as a standard [17].

Statistical analysis. All data are presented as the mean ± SEM of cAMP and cGMP in three aliquots of cells; each analysis was performed in duplicate. Statistical comparisons were carried out using Bartlett's test for heterogeneity of variance and Duncan's multiple range test [18].

RESULTS

Effects of ethanol on PE potentiation of VIP-stimulated cAMP and cGMP accumulation. As in our previous study, activation of α_1-adrenoceptors by PE potentiated the VIP-stimulated cAMP and cGMP responses 5- and 10-fold, respectively (Fig. 1) [11]. The inhibitory effects of ethanol on PE potentiation of VIP-stimulated cAMP and cGMP responses were concentration dependent with

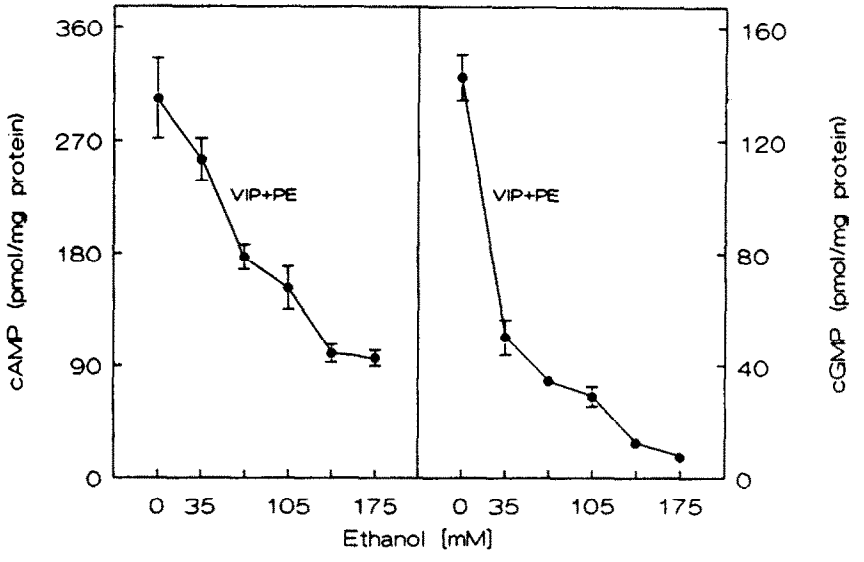

Fig. 1. Concentration responses of ethanol on PE potentiation of VIP-stimulated cAMP and cGMP accumulation. Graded concentrations of ethanol were added 15 sec before the addition of VIP (1 μM) and PE (10 μM). After 15 min, cells were pelleted and cyclic nucleotide content was determined. Each point is the mean ± SEM of triplicate samples assayed in duplicate. The absence of an error bar indicates that the SEM fell within the symbol. Control cAMP and cGMP values were 20 ± 0.5 and 2.0 ± 0.35 pmol/mg protein, respectively.

estimated IC_{50} values of 70 and 30 mM, respectively (Fig. 1, Table 1). Thus, compared to the cAMP response, the cGMP response appears to be more sensitive to the inhibitory action of ethanol.

Effects of ethanol on PMA potentiation of VIP-stimulated cAMP and cGMP accumulation. PMA, an activator of protein kinase C, potentiated the VIP-stimulated cAMP response 10-fold and the cGMP response 2-fold (Fig. 2). In the presence of ethanol, the PMA potentiation of VIP-stimulated cAMP response remained unchanged ($P > 0.05$ for all PMA concentrations). As reported previously, PMA had only a small stimulatory effect on the cGMP response [14]. The small increase in cGMP was inhibited with 175 mM ethanol ($P < 0.05$, Fig. 3).

Effects of ethanol on PMA and K^+ potentiation of VIP-stimulated cAMP and cGMP accumulation. Full potentiation of the cGMP response by PMA was achieved when cells were treated simultaneously with a depolarizing concentration of K^+ [14] (Fig. 4). The maximal concentration of ethanol used (175 mM) was found to have no effect on the PMA and K^+ potentiation of VIP-stimulated cAMP response ($P > 0.05$). In comparison, 105 mM ethanol reduced the corresponding cGMP response by 40% ($P < 0.005$). Therefore, the cAMP response potentiated by protein kinase C appears to be insensitive to the action of ethanol.

Effects of ethanol on K^+ potentiation of VIP-stimulated cAMP and cGMP accumulation. Depolarizing concentrations of K^+ potentiated the VIP-stimulated cAMP and cGMP responses 5- and 15-fold, respectively (Fig. 5). In the presence of ethanol (50 mM), the maximal K^+ potentiation of the VIP-stimulated cAMP and cGMP responses was reduced by 41 and 54%, respectively ($P < 0.005$ for both) (Fig. 5). The inhibitory effects of ethanol on the K^+ potentiation of VIP-stimulated cAMP and cGMP responses were concentration dependent with estimated IC_{50} values of 50 and 30 mM, respectively (Fig. 6, Table 1). These findings suggest that ethanol may inhibit either the voltage-dependent Ca^{2+} channel or steps distal to elevation of $[Ca^{2+}]_i$ that are of importance to the potentiation mechanism.

Effects of ethanol on A23187 potentiation of VIP-stimulated cAMP and cGMP accumulation. The Ca^{2+} ionophore A23187 potentiated the VIP-stimulated cAMP and cGMP responses 6- and 20-fold (Fig. 7). Ethanol (50 mM), while having no effect on the A23187 potentiation of VIP-stimulated cAMP response, significantly reduced the corresponding cGMP response ($P < 0.005$ for A23187 concentrations $\geq 1\ \mu M$) (Fig. 7). The inhibitory

Table 1. IC_{50} Values of ethanol on α_1-adrenergic-mediated potentiation of VIP-stimulated cAMP and cGMP responses

Treatment	Estimated IC_{50} values (mM)	
	cAMP	cGMP
VIP + PE	70	30
VIP + PMA	No effect*	175
VIP + K^+ + PMA	>175	125
VIP + K^+	50	30
VIP + A23187	>175	120

* No inhibition by 175 mM ethanol.

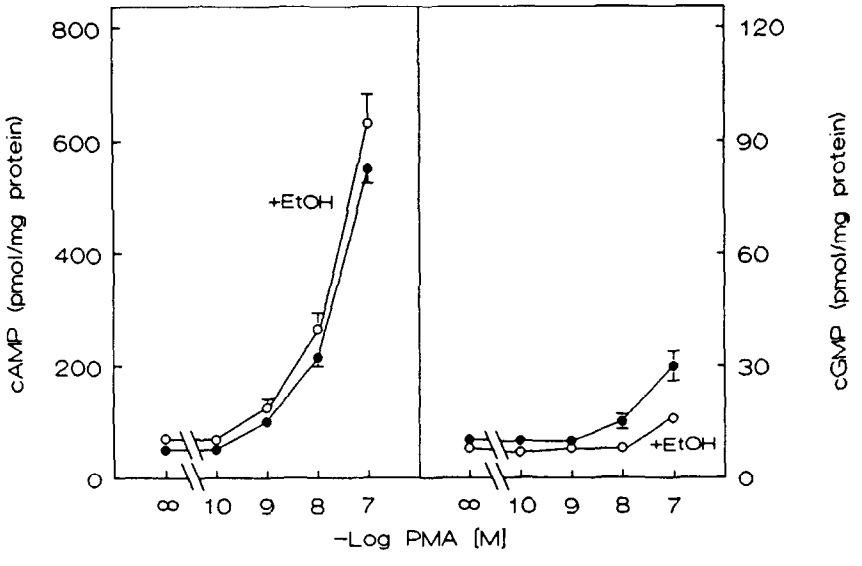

Fig. 2. Effects of ethanol on PMA potentiation of VIP stimulation of cAMP and cGMP accumulation. Ethanol (175 mM) was added 15 sec before the addition of VIP (1 μM) and graded concentrations of PMA. After 15 min, cells were pelleted for cyclic nucleotide determination. Each point is the mean ± SEM of triplicate samples assayed in duplicate.

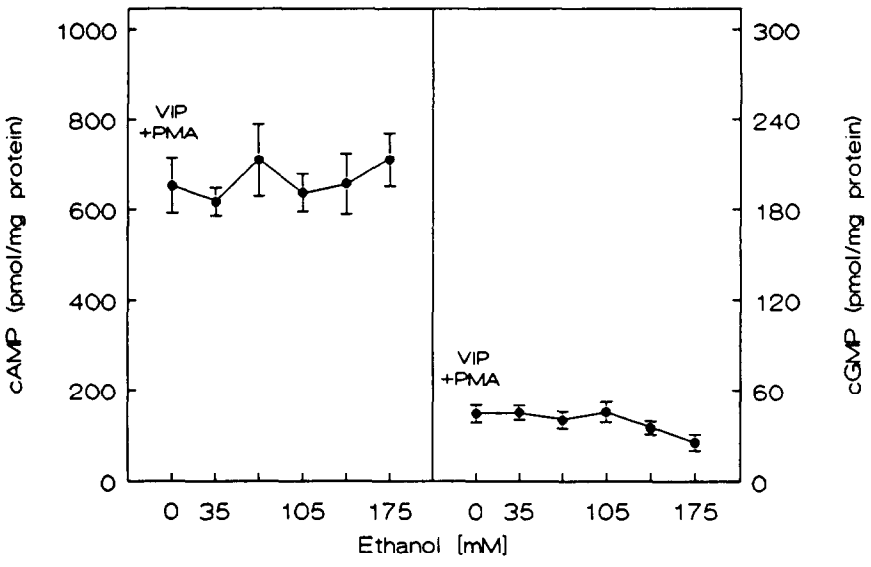

Fig. 3. Concentration responses of ethanol on PMA potentiation of VIP accumulation of cAMP and cGMP accumulation. Graded concentrations of ethanol were added 15 sec before the addition of VIP (1 μM) and PMA (0.1 μM). After 15 min, cells were pelleted for cyclic nucleotide determination. Each point is the mean ± SEM of triplicate samples assayed in duplicate.

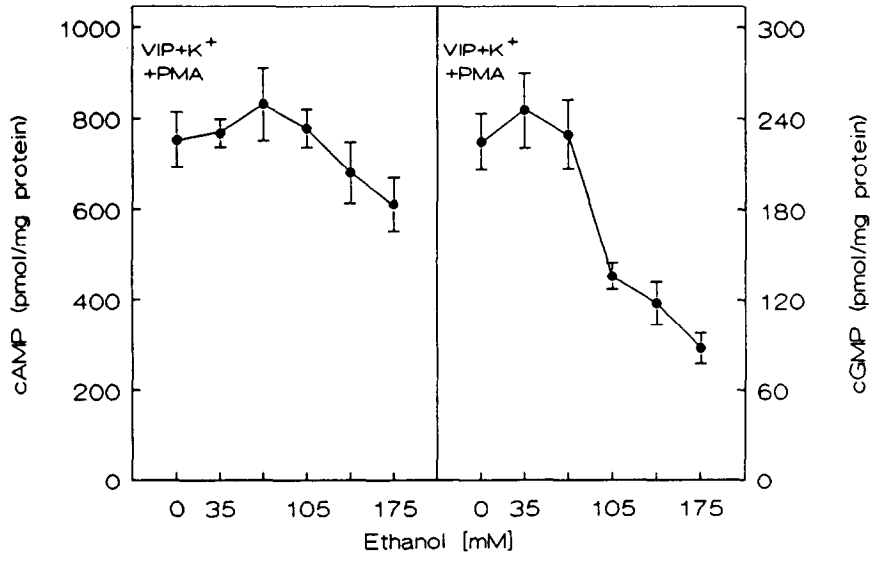

Fig. 4. Concentration responses of ethanol on PMA and K^+ potentiation of VIP stimulation of cAMP and cGMP accumulation. Graded concentrations of ethanol were added 15 sec before the addition of VIP (1 μM), K^+ (15 mM) and PMA (0.1 μM). After 15 min, cells were pelleted for cyclic nucleotide determination. Each point is the mean ± SEM of triplicate samples assayed in duplicate. Cyclic AMP values for VIP + K^+ and VIP + K^+ + 175 mM ethanol were 59 ± 7 and 84 ± 10 pmol/mg protein. The corresponding cGMP values were 17.5 ± 4.1 and 14.5 ± 2.9 pmol/mg protein, respectively.

effect of ethanol on the VIP and A23187 stimulated cGMP response was concentration dependent with an estimated IC_{50} value of 120 mM (Fig. 8, Table 1). In comparison, ethanol only inhibited the corresponding cAMP response at concentrations above 140 mM (Fig. 8).

DISCUSSION

In this study, two intracellular mechanisms that may account for the differential sensitivity between cAMP and cGMP responses to ethanol were examined. In the rat pineal transduction pathways,

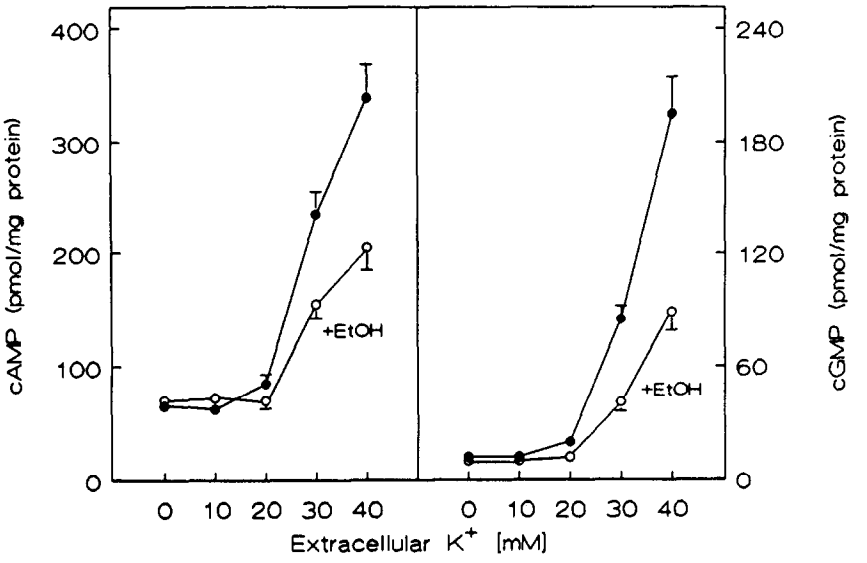

Fig. 5. Effects of ethanol on K^+ potentiation of VIP stimulation of cAMP and cGMP accumulation. Ethanol (50 mM) was added 15 sec before the addition of VIP (1 μM) and graded concentrations of K^+. After 15 min, cells were pelleted for cyclic nucleotide determination. Each point is the mean ± SEM of triplicate samples assayed in duplicate. The absence of an error bar indicates that the SEM fell within the symbol.

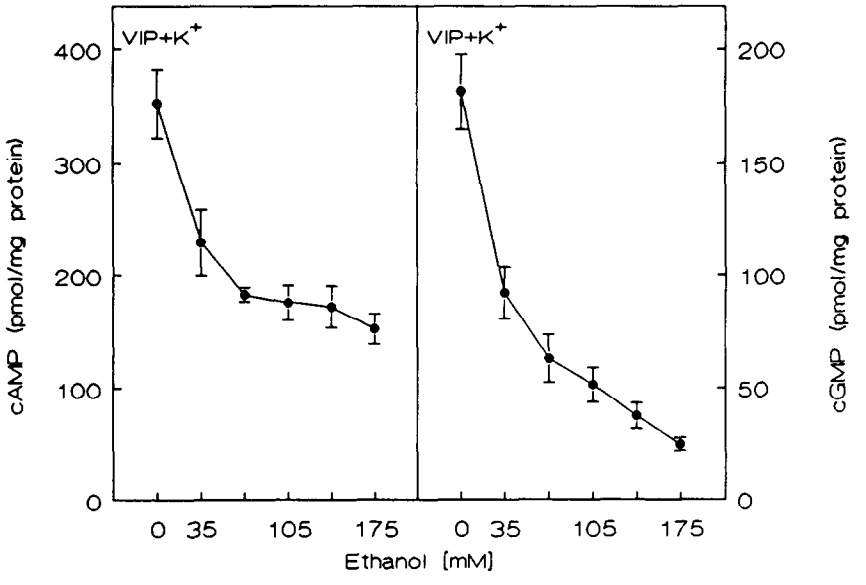

Fig. 6. Concentration responses of ethanol on K^+ potentiation of VIP stimulation of cAMP and cGMP accumulation. Graded concentrations of ethanol were added 15 sec before the addition of VIP (1 μM) and K^+ (40 mM). After 15 min, cells were pelleted for cyclic nucleotide determination. Each point is the mean ± SEM of triplicate samples assayed in duplicate.

α_1-adrenergic activation leads to activation of protein kinase C and elevation of $[Ca^{2+}]_i$ [19–21]. Both post-receptor events participate in the α_1-adrenergic potentiation of VIP-stimulated cyclic nucleotide responses [14]. In the case of cAMP, the α_1-adrenergic-mediated activation of protein kinase C [19, 21] appears to be sufficient for the potentiation mechanism. In comparison, full potentiation of the cGMP response by activation of protein kinase C requires the simultaneous elevation of $[Ca^{2+}]_i$ [14, 22]. Therefore, while PMA potentiated the VIP-stimulated cAMP response, its effect on the cGMP

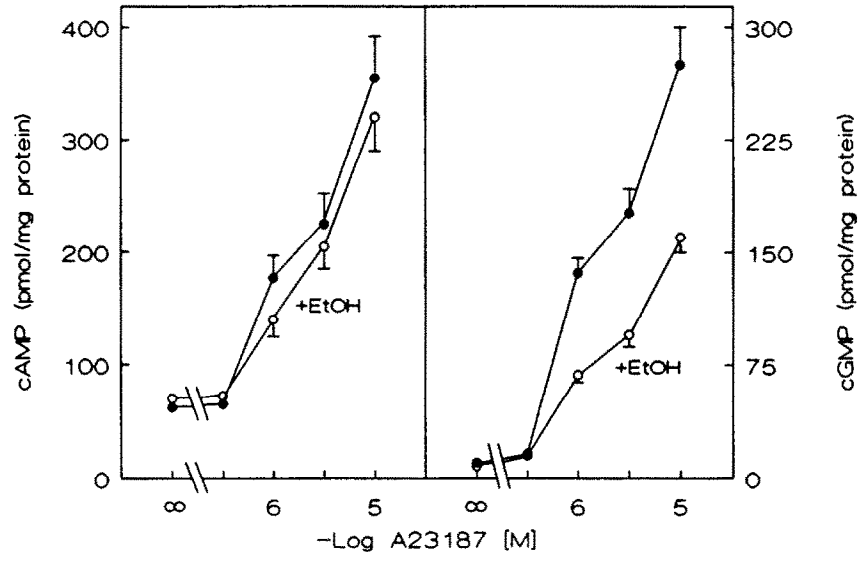

Fig. 7. Effects of ethanol on A23187 potentiation of VIP stimulation of cAMP and cGMP accumulation. Ethanol (50 mM) was added 15 sec before the addition of VIP (1 μM) and graded concentrations of A23187. After 15 min, cells were pelleted for cyclic nucleotide determination. Each point is the mean ± SEM of triplicate samples assayed in duplicate. The absence of an error bar indicates that the SEM fell within the symbol.

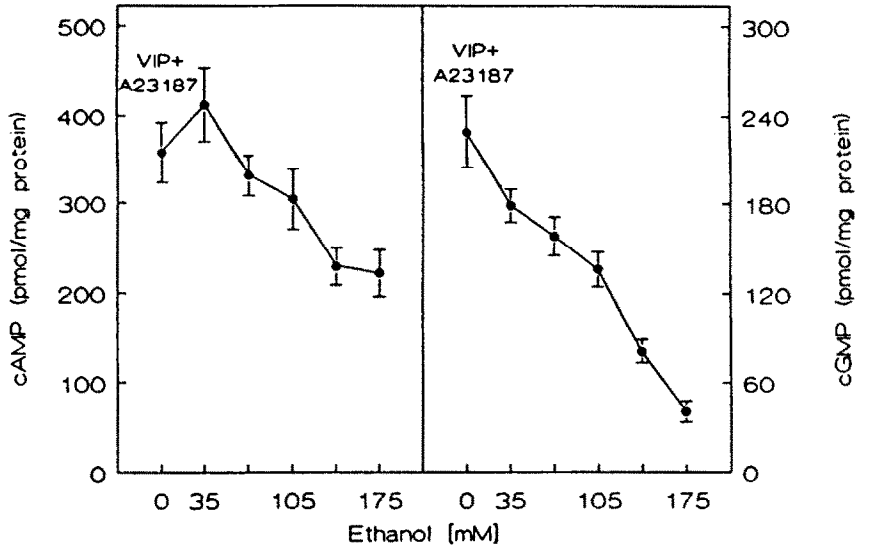

Fig. 8. Concentration responses of ethanol on A23187 potentiation of VIP stimulation of cAMP and cGMP accumulation. Graded concentrations of ethanol were added 15 sec before the addition of VIP (1 μM) and A23187 (10 μM). After 15 min, cells were pelleted for cyclic nucleotide determination. Each point is the mean ± SEM of triplicate samples assayed in duplicate.

response can only be observed in the presence of a depolarizing concentration of K^+ or other $[Ca^{2+}]_i$ elevating agents [14]. In contrast, agents which elevate $[Ca^{2+}]_i$, which have been shown to activate protein kinase C [21], are effective in potentiating the VIP-stimulated cAMP and cGMP responses.

As in our previous study, ethanol potently inhibited the PE potentiation of VIP-stimulated cAMP and cGMP response, with the cGMP response being more sensitive to the inhibitory action of ethanol ([2] Table 1). In the case of cAMP, we found that ethanol did not affect the PMA

potentiation of the VIP-stimulated cAMP response. In contrast, ethanol was effective in inhibiting the potentiating effects of PE and $[Ca^{2+}]_i$ elevating agents on VIP-stimulated cAMP responses. Since α_1-adrenergic activation leads to translocation of protein kinase C, these findings, taken together, suggest that ethanol has no effect on the potentiation of cAMP response by protein kinase C activation and that a Ca^{2+}-dependent event of importance to the potentiation mechanism is likely inhibited by ethanol. Consistent with this finding is the previously reported inhibitory effect of ethanol on Ca^{2+} influx in synaptosomes and PC12 cells [23–26].

Of the two Ca^{2+}-mediated potentiation of VIP-stimulated cAMP responses, compared to A23187, the potentiating effect by K^+ appears to be more sensitive to ethanol. Therefore, ethanol may have a direct effect on the voltage-dependent Ca^{2+} channels. Alternatively, steps distal to elevation of $[Ca^{2+}]_i$ may be affected by ethanol. Consistent with a direct effect of ethanol on the voltage-dependent Ca^{2+} channels is the recent finding that these channels are sensitive to acute ethanol exposure [27] at concentrations similar to those of the present study. This is also supported by our preliminary finding that 25 mM ethanol had an inhibitory effect on the L-type of Ca^{2+} channels in intact pinealocytes (our unpublished results). Since ethanol also inhibits the A23187 potentiation of VIP-stimulated cGMP and, to a lesser degree, the cAMP response, steps distal to elevation of $[Ca^{2+}]_i$ may also be inhibited by ethanol. Indeed, ethanol has no effect on the increases in $[Ca^{2+}]_i$ by 4-Br-A23187 as determined by fura-2, a fluorescent indicator of $[Ca^{2+}]_i$ (our unpublished results).

As for the cGMP response, ethanol was inhibitory to the potentiating effects by PE and $[Ca^{2+}]_i$ elevating agents. Compared to the cAMP responses, the cGMP responses appeared to be more sensitive to the inhibitory effects of ethanol. One possible explanation for the increased sensitivity of the cGMP response to ethanol treatment is a direct inhibitory action of ethanol on the guanylate cyclase activity. In several neural tissues, ethanol has an inhibitory action on the guanylate cyclase activity [28, 29]. However, even though inhibition of guanylate cyclase activity by ethanol may lead to a suppressed cGMP level, this is an unlikely explanation for the present finding since ethanol has no effect on VIP- or isoproterenol-stimulated cGMP response in the rat pineal gland [2]. Alternatively, the increased sensitivity may be a reflection of the increased Ca^{2+} dependency of the cGMP response [22]. Consistent with this hypothesis is the finding that the potentiated cGMP response by PMA and K^+ was inhibited by ethanol. In contrast, ethanol had no effect on the PMA and K^+ potentiation of the cAMP response, a finding consistent with our previous demonstration that activation of protein kinase C alone is sufficient for the potentiation of the cAMP response and that both protein kinase C and elevation of $[Ca^{2+}]_i$ are required for the potentiation of the cGMP response [22].

In summary, results from the present study have extended our previous knowledge of the mechanisms involved in the action of ethanol on α_1-adrenergic potentiation of VIP-stimulated cAMP and cGMP responses in rat pinealocytes. Ethanol appears to inhibit a Ca^{2+}dependent step that is critical for the α_1-adrenergic potentiation of VIP-stimulated cAMP and cGMP accumulation. This is based on the finding that ethanol had potent inhibitory actions on the potentiating effects of PE or $[Ca^{2+}]_i$ elevating agents on the VIP-stimulated cAMP and cGMP responses. In contrast, the protein kinase C pathway appears to be unaffected by ethanol treatment since ethanol had no effect on the PMA potentiation of VIP-stimulated cAMP response.

Acknowledgements—C. L. Chik is a clinical investigator of the Alberta Heritage Foundation for Medical Research (AHFMR) and A. K. Ho is a scholar of the Medical Research Council of Canada (MRC). This work was supported by grants from MRC and AHFMR to C. L. C. and A. K. H. We thank Marc Girard for his assistance with the preparation of the manuscript.

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