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EFFECT OF ETHANOL ON CYCLIC AMP LEVELS IN INTACT PC12 CELLS

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Abstract—Two subclones of the rat pheochromocytoma celline, PC12, were used to compare the effects of ethanol on adenylate cyclase activity in isolated membranes with its effects on cyclic AMP accumulation in intact cells. Consistent with previous reports, ethanol increased basal and 2-chloroadenosine-stimulated adenylate cyclase activity in isolated membrane preparations from both subclones. However, ethanol had opposite effects on agonist-stimulated cyclic AMP accumulation in intact cells of the two subclones, enhancing accumulation in one subclone, and inhibiting it in the other. The inhibition of cyclic AMP accumulation did not result from stimulation of phosphodiesterase activity, activation of the inhibitory guanyl nucleotide regulatory protein, G_i, or stimulation of protein kinase C. The results indicate that extrapolation of the effects of ethanol from one cell type to another, or from *in vitro* to *in vivo* systems, may be complicated by the interaction of ethanol with regulatory processes that influence second messenger systems, and can differ in various types of intact cells.

Approximately 20 years ago Gorman and Bitensky [1] observed that ethanol enhanced the activity of rat hepatic adenylate cyclase. Since that time, studies utilizing isolated membranes from a variety of tissues have confirmed the original observation and have gone on to examine the interaction of ethanol with adenylate cyclase at the molecular level [2–5]. These studies have indicated that the enhancement of adenylate cyclase activity by ethanol is mediated primarily by an action on the stimulatory guanyl nucleotide binding protein, G_s,‡ or the G_s–adenylate cyclase interaction. In contrast, ethanol has little effect on the catalytic subunit of adenylate cyclase or on the inhibitory regulation of adenylate cyclase [5–7].

Although the effects of ethanol on adenylate cyclase activity in isolated membrane preparations have been studied in some detail, many factors influence the levels of cyclic AMP within a cell [8–10], and it remains to be shown whether increases in adenylate cyclase activity are always reflected in increases in cyclic AMP content of intact cells. While, in some instances, ethanol has been reported to increase cellular cyclic AMP content [11, 12], few investigations have compared the effects of ethanol in isolated membranes to those in whole cell preparations. In one report [13], ethanol was found to increase PGE_1-stimulated [^{3}H]cyclic AMP formation from [^{3}H]ATP in intact N1E-115 cells to a much greater extent than would have been expected, based on the ethanol-induced increase in PGE_1-stimulated adenylate cyclase activity in membranes from the same cells. To further evaluate this relationship, we have examined, in the present study, the effects of ethanol on adenylate cyclase activity in isolated membranes and on cyclic AMP levels in intact cells of two subclones of the rat pheochromocytoma cell line, PC12.

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‡ Abbreviations: G_s, stimulatory guanyl nucleotide binding protein; G_i, inhibitory guanyl nucleotide binding protein; ADA, adenosine deaminase; 2ClAdo, 2-chloroadenosine; GppNHp, 5′-guanylylimidodiphosphate; PDBu, phorbol-12,13-dibutyrate; VIP, vasoactive intestinal peptide; PGE_1, prostaglandin E_1; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; RIA, radio-immunoassay; and ANOVA, one-way analysis of variance.

MATERIALS AND METHODS

Tissue culture. One subclone of PC12 cells ("$PC12_{Rabe}$") was obtained originally from Dr. Lloyd Greene and was subcultured in our laboratory, as previously described [14], in medium containing 10% horse serum (GIBCO, Grand Island, NY) and 5% fetal bovine serum (K.C. Biologicals, Lenexa, KS). The other subclone of PC12 cells was obtained from Dr. John Daly (NIDDKD, Bethesda, MD) ("$PC12_{Daly}$"). The $PC12_{Daly}$ cells were grown in our laboratory similarly to the $PC12_{Rabe}$ cells, except that the medium contained 6% horse serum and 6% fetal bovine serum (GIBCO) plus 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma Chemical Co., St. Louis, MO) [8].

Cell viability was assessed by trypan blue exclusion and was >95%. When compared to control cells, cells that had been treated with 400 mM ethanol for 1 hr did not show any difference in viability. In addition, consistent responses were obtained over 50+ passages. The lowest passage of $PC12_{Rabe}$ was passage 35; the lowest $PC12_{Daly}$ passage was 21.

Adenylate cyclase assay. Adenylate cyclase activity was measured in washed cell membranes (50–100 μg protein/assay) by assessing the conversion of [α-^{32}P]ATP (NEN-Dupont, Boston, MA) to [^{32}P]cyclic

AMP, as described by Saito *et al.* [5], except that the incubation mixture contained 0.5 mM Ro 20-1724 instead of theophylline. The incubation mixture, containing the concentrations of GppNHp, 2-chloroadenosine (2ClAdo) and/or ethanol indicated in the Results, was kept at 37° for 5 min before initiation of the reaction with the addition of cell membranes. After 10 min, incubations were terminated as described [5], and [^{32}P]cyclic AMP was isolated on Dowex and alumina columns by the method of Salomon *et al.* [15]. Protein content was determined by the method of Lowry *et al.* [16] using bovine serum albumin as a standard.

Cyclic AMP content of intact cells. For assay of cellular cyclic AMP, cells were centrifuged to remove growth medium and were resuspended in ice-cold HEPES-buffered saline (HBS) [14]. Where indicated, adenosine deaminase (ADA, 1 μg/mL final concentration, Boehringer Mannheim, Indianapolis, IN) was also present in the resuspension buffer. Aliquots of cells (200–400 μg protein/assay) were added to assay tubes which were placed in a 37° shaking waterbath. At this point, in certain assays, the phosphodiesterase inhibitor Ro 20-1724 (0.5 mM final concentration), or Ro 20-1724 plus staurosporine (1 μM final concentration, Boehringer Mannheim, Indianapolis, IN) were added. After 5 min, prewarmed HBS alone or HBS containing drugs, as indicated in the Results, was added to each tube and reactions were allowed to proceed for 10 min. Reactions were stopped by the addition of perchloric acid (PCA, 0.5 M final concentration), and PCA was precipitated at 4° using a sufficient volume of 15% KOH/15% $KHCO_3$ to leave the pH of the supernatant fraction at approximately 7. Cyclic AMP levels in the supernatant fraction were determined by radioimmunoassay [17] using [^{125}I]cyclic AMP (220 Ci/mmol, Hazelton, Vienna, VA) and antiserum provided by Dr. Larry Tamarkin (NIMH, Bethesda, MD). The PCA-precipitated cell pellet was solubilized in 1 M NaOH for protein determinations. The vehicle used to dissolve the phorbol esters and forskolin (dimethyl sulfoxide, 0.002% final concentration) had no effect on basal or agonist-stimulated cyclic AMP accumulation in either the absence or presence of ethanol.

[^{3}H]*Cyclic AMP formation in intact cells.* Cell adenine nucleotides were labeled by adding 5 μCi/mL of [^{3}H]adenine (27 Ci/mmol, Amersham, Arlington Heights, IL) to each flask of cells and incubating for 1 hr at 37°. Cells were then centrifuged at 500 *g* for 10 min, and growth medium containing unincorporated [^{3}H]adenine was aspirated. The cell pellet was resuspended in ice-cold HBS, and assays were performed as described above for measurement of cyclic AMP levels in intact cells. In these assays, the PCA-soluble material was neutralizing using 15% KOH/15% KH_2PO_4. [^{3}H]Cyclic AMP in the supernatant fraction was determined using the chromatographic method of Salomon *et al.* [15].

Pertussis toxin treatment. Cells were incubated with pertussis toxin (200 ng/mL, final concentration, List Biological Laboratories, Campbell, CA) in growth medium overnight. Growth medium was removed and cells were washed with HBS as described above prior to the determination of cyclic AMP content or [^{3}H]cyclic AMP accumulation.

Data expression. In all experiments, data are expressed as means ± SE, and basal values have been subtracted from stimulated values.

Materials. 2-Chloroadenosine and muscarine were obtained from Sigma. Forskolin, phorbol-12,13-dibutyrate and 4α-phorbol-12,13-didecanoate were obtained from Calbiochem (La Jolla, CA). Vasoactive intestinal peptide was obtained from Bachem (Torrance, CA). Ro 20-1724 was a gift from Dr. Peter Sorter (Hoffman-La Roche, Nutley, NJ).

RESULTS

Effects of ethanol on adenylate cyclase activity in cell membrane preparations. Basal and agonist-stimulated adenylate cyclase activities were higher in $PC12_{Daly}$ (Fig. 1A) than in $PC12_{Rabe}$ cells (Fig. 1B). Ethanol produced a concentration-dependent increase in basal and agonist-stimulated adenylate cyclase activity in membranes isolated from either subclone (Fig. 1, A and B). In both subclones, the threshold for ethanol-mediated stimulation of basal adenylate cyclase activity was approximately 200 mM, whereas the threshold for stimulation of GppNHp- or 2ClAdo plus GppNHp-stimulated adenylate cyclase activity was approximately 50 and 100 mM ethanol respectively.

Effects of ethanol on cyclic AMP levels and [^{3}H]*cyclic AMP formation in intact cells.* Although ethanol enhanced adenylate cyclase activity in cell membranes from both subclones of PC12 cells, ethanol enhanced 2ClAdo-stimulated cyclic AMP accumulation only in intact $PC12_{Daly}$ cells (Fig. 1C). In intact cells of the $PC12_{Rabe}$ subclone, ethanol *inhibited* both basal and 2ClAdo-stimulated cyclic AMP accumulation (Fig. 1D). Ethanol also inhibited VIP- and forskolin-stimulated cyclic AMP accumulation in these cells (Fig. 1E). A similar inhibition by ethanol in $PC12_{Rabe}$ cells was observed when cells were loaded with [^{3}H]adenine, and stimulation of cellular [^{3}H]cyclic AMP accumulation by 2ClAdo or VIP was measured. Ethanol (200 mM) significantly ($P < 0.01$, Student's *t*-test) reduced 2ClAdo (1 μM)-stimulated cyclic AMP generation from 36.5 ± 0.8 to 24.5 ± 0.3 cpm × 10^3/mg protein/10 min ($N = 8$) and VIP (1 μM)-stimulated cyclic AMP generation from 50.0 ± 2.2 to 37.1 ± 3.2 cpm × 10^3/mg protein/10 min ($N = 4$ and 6 respectively). Inhibition of a magnitude similar to that produced by 200 mM ethanol was observed when 10 mM butanol was substituted for ethanol in these experiments (data not shown).

In addition to their distinct responses to ethanol, several other differences between the intact cells of the two subclones were observed. The accumulation of cyclic AMP in $PC12_{Daly}$ cells was insensitive to VIP at concentrations as high as 10 μM. Furthermore, a concentration of 10 μM 2ClAdo was necessary to obtain a substantial elevation of cellular cyclic AMP levels in $PC12_{Daly}$ cells, as previously reported [8], whereas 1 μM 2ClAdo caused an approximately 6-fold increase in cyclic AMP levels in $PC12_{Rabe}$ cells (Fig. 1). It was also necessary to treat $PC12_{Daly}$ cells with ADA and Ro 20-1724 in order to observe

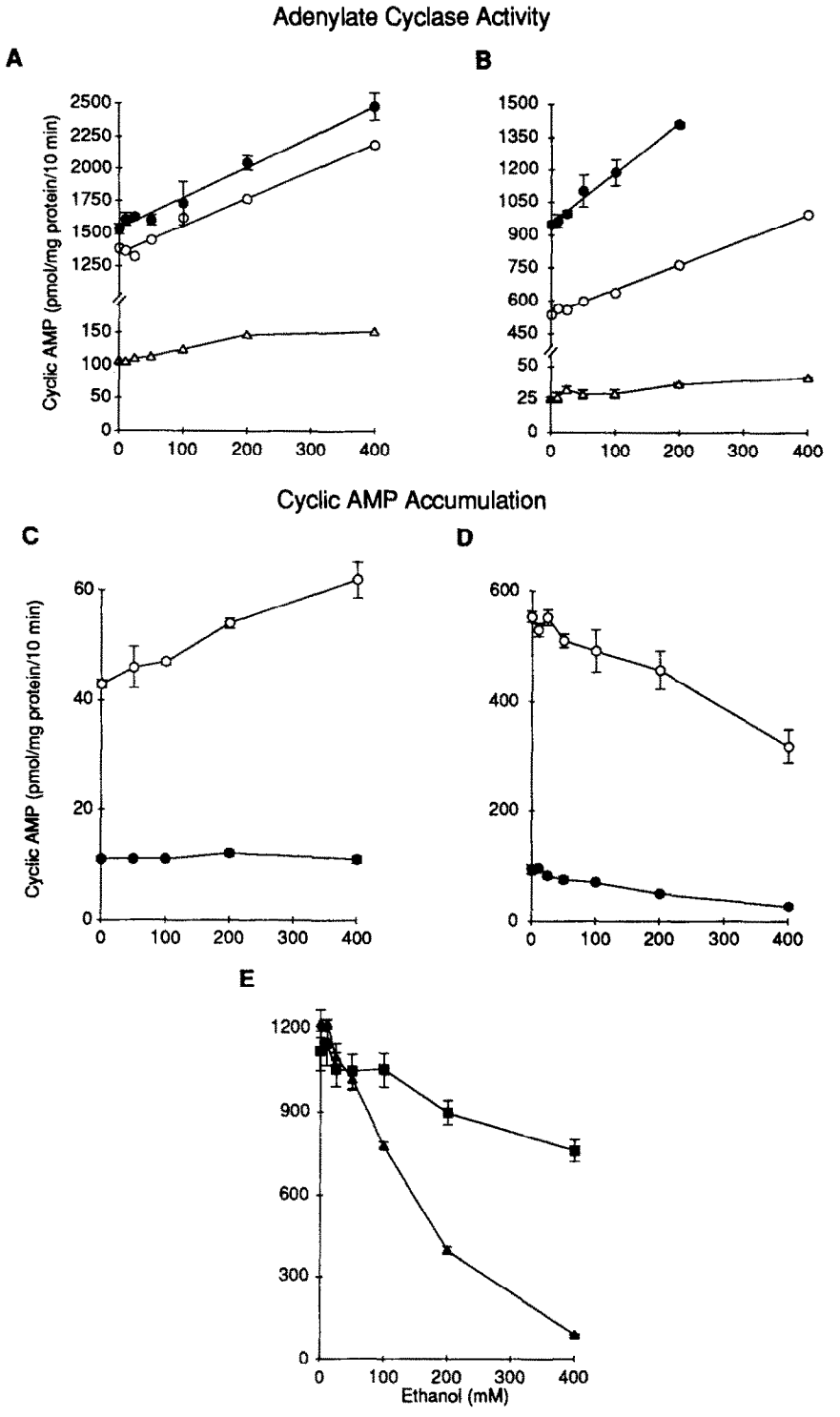

Fig. 1. Comparison of the effects of ethanol on adenylate cyclase activity and cyclic AMP accumulation in two subclones of PC12 cells. Adenylate cyclase activity in membranes of $PC12_{Daly}$ (A) or $PC12_{Rabe}$ (B) cells was measured as described in Materials and Methods. No additions (△), 10 μM GppNHp (○) or 10 μM GppNHp and 1 μM 2ClAdo (●) were present in the reaction buffer. Values are the means ± SE of triplicate determinations in a representative assay; each assay was repeated twice. Cyclic AMP accumulation in intact $PC12_{Daly}$ (C) or $PC12_{Rabe}$ (D and E) cells was determined by radioimmunoassay as described in Materials and Methods. $PC12_{Daly}$ cells (C) were resuspended in HEPES-buffered saline containing 1 μg/mL adenosine deaminase (final concentration). Following a 5-min preincubation in Ro 20-1724 (0.5 mM, final concentration), cells were incubated with either 10 μM 2ClAdo (○) or vehicle (●) plus the concentration of ethanol indicated. Values are means ± SE, N = 4–6. $PC12_{Rabe}$ cells (D and E) were resuspended in HEPES-buffered saline and were preincubated for 5 min prior to the addition of 1 μM 2ClAdo (○), 1 μM VIP (■), 1 μM forskolin (▲) or no drug (●), with or without ethanol. Cells were incubated for an additional 10 min. Values are means ± SE, N = 4.

Table 1. Effect of pertussis toxin treatment on the ability of ethanol to inhibit cyclic AMP accumulation in intact PC_{Rabe} cells

Condition	Cyclic AMP (pmol/mg protein/10 min)	
	Control	Pertussis toxin-treated
2ClAdo	668 ± 53	669 ± 35
2ClAdo + ethanol	479 ± 38*	425 ± 19*
VIP	1509 ± 146	1724 ± 161
VIP + ethanol	1233 ± 65*	1419 ± 59*
Forskolin	2139 ± 166	2138 ± 109
Forskolin + muscarine	1576 ± 98*	2192 ± 83

Pertussis toxin-treated cells were incubated overnight in growth medium containing pertussis toxin (200 ng/mL) prior to the experiment. After resuspension in HEPES-buffered saline, all cells were preincubated with 0.5 mM Ro 20-1724 for 5 min prior to addition of the drugs indicated. The concentration of ethanol was 200 mM, the concentration of muscarine was 100 μM, and other compounds were 1 μM. All reactions were stopped after 10 min, and cyclic AMP levels determined by RIA. Values are the means ± SE from four determinations.

* Significantly different from the respective value in the absence of ethanol or muscarine, $P < 0.05$ (Student's t-test).

stimulation of cyclic AMP accumulation by 2ClAdo. As seen in Fig. 1D, these treatments were not necessary in $PC12_{Rabe}$ cells. However, the inhibitory effect of ethanol on 2ClAdo-stimulated cyclic AMP accumulation in $PC12_{Rabe}$ cells was unchanged by treatment with ADA and Ro 20-1724 (2ClAdo, 1 μM: 725 ± 24; 2ClAdo plus ethanol, 100 mM: 606 ± 42 pmol/mg protein/10 min; N = 4 per group). On the other hand, pretreatment of these cells with ADA reduced "basal" cyclic AMP levels and eliminated the inhibition of "basal" cyclic AMP accumulation by ethanol, suggesting that this latter effect actually represented inhibition of cyclic AMP accumulation that was stimulated by endogenous adenosine.

Effect of pertussis toxin pretreatment ($PC12_{Rabe}$). To determine whether the inhibitory guanyl nucleotide regulatory protein, G_i, might be involved in the ethanol-induced lowering of cellular cyclic AMP levels in $PC12_{Rabe}$ cells, the effect of pertussis toxin was tested. Although pertussis toxin treatment completely blocked the ability of muscarine (an agent known to inhibit adenylate cyclase activity through activation of G_i [18]) to inhibit forskolin-stimulated cyclic AMP accumulation in $PC12_{Rabe}$ cells, the ability of ethanol to inhibit agonist-stimulated cyclic AMP accumulation was unaffected (Table 1).

Effects of phorbol esters and a protein kinase C inhibitor on cyclic AMP levels ($PC12_{Rabe}$). Phorbol-12,13-dibutyrate (PDBu) significantly inhibited cyclic AMP accumulation produced by treatment of $PC12_{Rabe}$ cells with 2ClAdo or VIP, but did not inhibit forskolin-stimulated cyclic AMP accumulation (Fig. 2). When cells were incubated with phorbol ester plus ethanol, the inhibition of cyclic AMP accumulation was roughly additive (Fig. 2). The inactive phorbol ester, 4α-phorbol-12,13-didecanoate, had no effect on the ability of agonists to raise cyclic AMP levels (Fig. 2). Because of similarities in the effect of the active phorbol ester and ethanol on cyclic AMP accumulation, it seemed possible that these agents might share a common mechanism of action, such as stimulation of protein kinase C activity. However, pretreatment of $PC12_{Rabe}$ cells with the putative protein kinase C inhibitor, staurosporine, had no effect on the inhibition on cyclic AMP accumulation produced by ethanol, although this treatment reversed the inhibition produced by PDBu (Table 2).

DISCUSSION

Although ethanol increases receptor-activated adenylate cyclase activity in most membrane preparations [1–3, 5], the results presented here indicate that the effect of ethanol on adenylate cyclase in isolated membranes does not necessarily predict either the *magnitude* or the *direction* of the effects of ethanol on cyclic AMP levels in intact cells. Ethanol enhanced both 2ClAdo-stimulated adenylate cyclase activity in membranes and cyclic AMP accumulation in intact PC12 cells from the laboratory of Dr. John Daly ($PC12_{Daly}$). However, while ethanol also enhanced membrane adenylate cyclase activity in the subclone of PC12 cells from our laboratory, $PC12_{Rabe}$, similar concentrations of ethanol clearly reduced agonist-stimulated cyclic AMP accumulation in intact cells. The same discrepancy was noted whether, in intact $PC12_{Rabe}$ cells, cyclic AMP levels or the formation of [^{3}H]cyclic AMP from [^{3}H]ATP (i.e. cyclic AMP production) was measured.

To investigate the mechanism which might regulate the response of the intact cell to ethanol, several experiments were performed utilizing the $PC12_{Rabe}$ subclone. The ability of a much lower concentration (10 mM) of butanol to inhibit [^{3}H]cyclic AMP accumulation to the same extent as that observed with 200 mM ethanol demonstrates that the inhibition was not due to an osmotic effect of ethanol. The reduction of cyclic AMP levels in intact $PC12_{Rabe}$

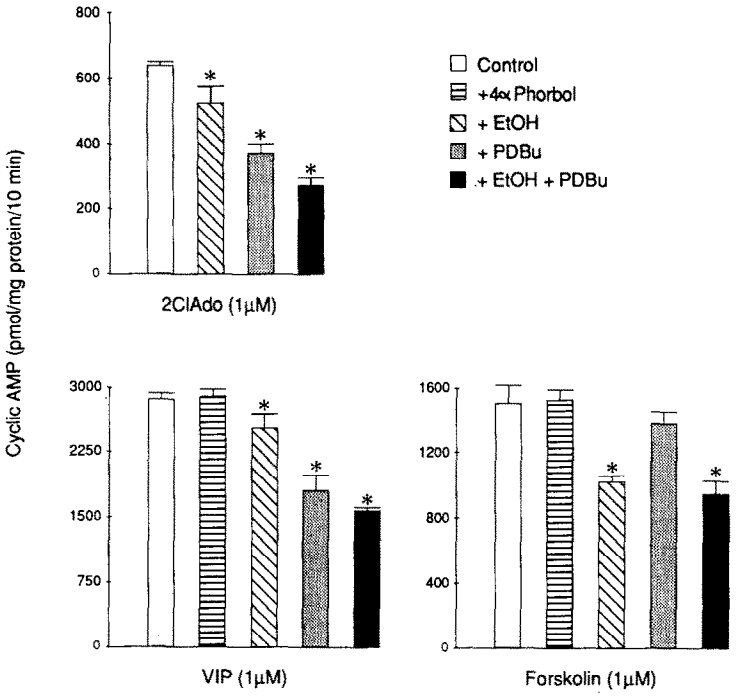

Fig. 2. Comparison of the effects of ethanol and phorbol esters on cyclic AMP accumulation in PC_{Rabe} cells. $PC12_{Rabe}$ cells were preincubated for 5 min with Ro 20-1724 (0.5 mM, final concentration) and were then incubated in the presence of the drugs indicated for 10 min. The concentration of 4α-phorbol-12,13-didecanoate (4α-Phorbol) was 1 μM, PDBu was 100 nM, and ethanol was 100 mM. Cyclic AMP levels were determined by radioimmunoassay. Basal values have been subtracted. Values are means ± SE, N = 4. Key: * $P < 0.05$, compared to control (ANOVA and Student's *t*-test).

Table 2. Effect of staurosporine on ethanol- and phorbol dibutyrate-mediated inhibition of cyclic AMP accumulation in intact $PC12_{Rabe}$ cells

	Cyclic AMP (pmol/mg protein/10 min)	
Condition	Control	Staurosporine-treated
2ClAdo	307 ± 27	275 ± 36
2ClAdo + ethanol (400 mM)	140 ± 11*	124 ± 15*
2ClAdo + PDBu (100 nM)	225 ± 13*	242 ± 14

Cells were preincubated for 5 min with 0.5 mM Ro 20-1724 with or without 1 μM staurosporine. Drugs were then added, and incubations were terminated 10 min later. Cyclic AMP levels were determined by RIA. Values are the means ± SE from four determinations.

* Significantly different from the respective 2ClAdo-stimulated values, $P < 0.05$ (ANOVA and Student's *t*-test).

cells did not appear to be due to an ethanol-mediated increase in phosphodiesterase activity, since the effect was not reversed by the phosphodiesterase inhibitor, Ro 20-1724. This finding is consistent with earlier reports that ethanol has little effect on phosphodiesterase activity [19, 20]. Furthermore, the inhibition of cyclic AMP accumulation did not appear to involve ethanol-mediated activation of the inhibitory guanyl nucleotide regulatory protein, G_i. Pertussis toxin catalyzes the ADP-ribosylation of the α subunit of G_i, preventing G_i-mediated inhibition of adenylate cyclase activation [21]. Although pertussis toxin treatment blocked muscarine-induced inhibition of cyclic AMP accumulation, as expected, this treatment had no effect on the reduction of 2ClAdo- and VIP-stimulated cyclic AMP levels caused by ethanol. The lack of G_i involvement is consistent with studies in brain membranes which indicated that ethanol does not alter agonist-induced inhibition of adenylate cyclase activity [6, 7]. More recent studies, using membranes from Cyc^- S49 cells, also showed that G_i is not involved in ethanol-induced inhibition of forskolin-stimulated adenylate cyclase activity [22]. These results, however, do not preclude the

possibility that ethanol may cause inhibition of agonist-stimulated cyclic AMP accumulation in intact PC12 cells by raising levels of β/γ subunits from another, as yet unidentified, guanyl nucleotide regulatory protein in the cell, since these subunits also may act as inhibitors of adenylate cyclase [23].

Activation of protein kinase C has been reported to have inhibitory effects on adenylate cyclase activity in a variety of tissues [9, 24–27]. Since phorbol esters inhibited receptor-mediated cyclic AMP accumulation in $PC12_{Rabe}$ cells, it was possible that the inhibitory effect of ethanol might also be mediated through protein kinase C activation, either as a result of phosphatidylinositol bisphosphate hydrolysis [28] or by an increase in intracellular free Ca^{2+} [28–30]. However, two of our observations indicated that the inhibition of cyclic AMP accumulation by ethanol was not mediated by activation of protein kinase C. First, ethanol inhibited forskolin-stimulated cyclic AMP accumulation, whereas phorbol esters did not. It might be argued that the effect of ethanol on forskolin-stimulated cyclic AMP accumulation represents a unique situation, since high concentrations of ethanol have been reported to inhibit forskolin-stimulated adenylate cyclase activity in membrane preparations [31–33]. However, the low concentrations of ethanol used in the present study would not substantially lower forskolin-stimulated adenylate cyclase activity in a membrane preparation [31].

More compelling evidence against a role of protein kinase C in the inhibitory effect of ethanol was the finding that the putative protein kinase C inhibitor staurosporine, which blocked phorbol ester-mediated reduction of cyclic AMP accumulation, did not alter ethanol-mediated reduction of cyclic AMP levels. If ethanol reduces cyclic AMP levels through a protein kinase C-like mechanism, it must be acting at a step subsequent to protein kinase C activation or by an action on another protein kinase.

Chik *et al.* [34] recently reported that ethanol inhibits agonist-stimulated cyclic AMP accumulation in pinealocytes, but only in the case where VIP-stimulated cyclic AMP accumulation was potentiated by simultaneous stimulation with phenylephrine. In the absence of concomitant phenylephrine stimulation, ethanol potentiated VIP stimulation of cyclic AMP accumulation. The intact cells of the $PC12_{Rabe}$ subclone had higher basal and agonist-stimulated levels of cyclic AMP and were more sensitive to agonist stimulation of cyclic AMP accumulation than $PC12_{Daly}$ cells. It is possible that some endogenous characteristic of $PC12_{Rabe}$ cells results in an effect on agonist-stimulated cyclic AMP accumulation that is similar to the phenylephrine-induced potentiation of the VIP response observed in pinealocytes. Thus, like the pinealocytes, when $PC12_{Rabe}$ cells are stimulated with agonist, the cyclic AMP response of the cells is vulnerable to inhibition by ethanol.

At present it is unknown whether the two subclones of PC12 cells differ genetically or whether the difference in response to ethanol arises from culture conditions, which may influence the second messenger systems in the cells (e.g. see Ref. 35). Another possibility is that cells of the two subclones were assayed during different stages of growth, which were not evaluated in these studies. In any case, our results demonstrate that an increase in adenylate cyclase activity in isolated membranes cannot necessarily be extrapolated to an effect on cyclic AMP levels in intact cells. Furthermore, the finding that ethanol can produce opposite effects on cyclic AMP levels in two seemingly similar *in vitro* cell culture systems indicates that results obtained in such systems will not always reflect the *in vivo* situation. On the other hand, the important influence of cellular regulatory mechanisms on the response to ethanol may well contribute to the diversity of the *in vivo* actions of ethanol.

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