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INHIBITION BY ETHANOL OF FORSKOLIN-STIMULATED ADENYLATE CYCLASE IN A MURINE NEUROBLASTOMA CLONE (N1E-115)

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Abstract—Forskolin, a diterpene activator of adenylate cyclase, stimulated the formation of cyclic AMP in intact murine neuroblastoma clone N1E-115 cells and stimulated adenylate cyclase activity in a membranal preparation from these cells. Ethanol caused a concentration-dependent inhibition of the forskolin-stimulated responses in both preparations. In intact cells, the inhibition appeared to be non-competitive. However, in the membranal preparation the inhibition was more of a competitive nature. In addition, there was also a large difference in the amount of inhibition in the two systems. Thus, the inhibition by ethanol was nearly twice as much with intact cells as with membranes. Sucrose appeared to mimic these effects of ethanol, suggesting that with intact cells the effect of this alcohol may be due, in part, to changes in cellular osmotic pressure.

Forskolin, a diterpene isolated from the roots of *Coleus forskohli*, was first shown to be an activator of adenylate cyclase by Metzger and Lindner [1] who used rabbit heart slices. It has since been shown to stimulate adenylate cyclase in a wide variety of tissues, both in membranes and in intact cells [2]. It also has the unique ability to augment receptor-mediated stimulation of adenylate cyclase [3]. The mechanism of activation of adenylate cyclase by forskolin is still disputed. Although forskolin can activate adenylate cyclase in the absence of guanine nucleotides, recent evidence suggests that there are coupling proteins involved in the forskolin activation of adenylate cyclase [2, 4, 5].

Ethanol has also been shown to have a stimulatory effect on adenylate cyclase. In membranal preparations of various tissues, ethanol has stimulatory effects upon basal activity and activity stimulated by sodium fluoride, guanosine 5′-(β-γ-imido) triphosphate, cholera toxin and receptors [6, 7]. In intact cell systems, ethanol has little or no effect on basal cyclic AMP levels but causes a pronounced potentiation of receptor-mediated accumulation [8–11]. The mechanism of action of ethanol on adenylate cyclase is unclear. Several groups have shown that ethanol causes an increase in membrane fluidity, suggesting that perhaps this fluidity change would allow for a more efficient coupling between the subunits of adenylate cyclase [12–14]. However, a recent report by Hoffman and Tabakoff [15] suggests that ethanol acts directly upon the catalytic unit or the associated regulatory proteins.

Recently, it was reported that ethanol inhibits forskolin-stimulated adenylate cyclase in membranes from bovine corpora lutea and rat heart [16, 17]. This result is surprising, since generally any treatment which activates adenylate cyclase is potentiated by ethanol. Therefore, we decided to investigate further this finding using the well-characterized murine neuroblastoma clone N1E-115 [18]. The two reports dealing with forskolin inhibition by ethanol were both done with membranal preparations. In this report we investigated the effects using both membranes and intact cells.

METHODS AND MATERIALS

Cell culture. Murine neuroblastoma clone N1E-115 cells were grown as previously described [19]. Confluent cells were detached from flasks with Puck's D_1 solution and collected by low speed centrifugation (250 *g*). Cells to be used in intact cell experiments and labeled with [^{3}H]adenine were washed once and suspended in a physiological iso-osmolar phosphate-buffered saline solution (solution I) containing glucose and sucrose (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose, 2 mM Na_2HPO_4; pH adjusted to 7.35; osmolality adjusted to 340 ± 5 mOsm with sucrose). Cells to be used for the assay of membranal adenylate cyclase activity were washed and suspended in ice-cold buffer (solution II) containing 10 mM Tris–HCl, 0.32 M sucrose and 1.0 mM $MgCl_2$ (pH 7.40 at 30°).

Assay of cyclic [^{3}H]AMP levels in intact N1E-115 cells. Detached cells suspended in 3 ml of solution I (approximately 2–5 × 10^{-6} cells/ml) were transferred to a 25-ml Erlenmeyer flask to which was added 30 μCi of [^{3}H]adenine (Amersham, 22 Ci/mmole). The cells were then put in a shaking water bath at 37° for 45 min. The cells labeled with [^{3}H]-adenine were then assayed for cyclic [^{3}H]AMP formation as previously described [9]. Briefly, after labeling, the cells were diluted to about 5 × 10^5 cells/ml with solution I containing 0.3 to 4 mM 3-isobutyl-1-methylxanthine (IBMX) at 37° and distributed in

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320 μl aliquots to the wells of a multi-well tray at 37° in the shaker bath. The cells were allowed to equilibrate for 5–10 min before the compounds to be tested [ethanol, forskolin or prostaglandin E_1 (PGE_1)] were added in an 80 μl volume to give a final volume of 400 μl. After 10 min the assay was stopped by adding 30 μl of 50% (w/v) trichloroacetic acid (TCA) to each well. The cyclic [^{3}H]AMP levels were then determined as previously described [9].

Assay of adenylate cyclase activity in membranes. Detached cells suspended in approximately 5 ml of solution I were homogenized with approximately twenty strokes of a motor-driven Teflon–glass tissue grinder. The resulting homogenate was then centrifuged at 250 *g* for 1 min. The supernatant fraction was removed and centrifuged at 40,000 *g* for 30 min in a Sorvall RC-5B superspeed centrifuge at 4°. The pellet was suspended in solution II and centrifuged again at 40,000 *g*. After repeating this last step, the final washed pellet was resuspended in solution II to 2.5 mg protein/ml and stored above liquid nitrogen in 1 ml aliquots until use. Adenylate cyclase activity was determined by measuring the conversion of [^{3}H]-AMP to cyclic [^{3}H]AMP. The assay was carried out in a final volume of 100 μl containing 45 mM Tris–HCl (pH 7.40 at 30°), 5 mM $MgCl_2$, 20 mM creatine phosphate, 5 units of creatine phosphokinase, 0.3 mM IBMX, 1 mM cyclic AMP, 0.4 mM ATP, 0.1 mM GTP, 2 μCi [^{3}H]ATP, 25–50 μg protein (homogenate) and other compounds as indicated. This assay was terminated after 10 min with 30 μl of 50% TCA. The cyclic [^{3}H]AMP formed was isolated as previously described [9].

Materials. Forskolin was obtained from the Calbiochem-Behring Corp. (San Diego, CA). It was stored as a stock solution of 20 mM in absolute ethanol at −20°. At the highest concentration used in this study, 20 μM, the concentration of ethanol was approximately 20 mM, and all concentrations of ethanol reported are in addition to this amount. When concentrations greater than 20 μM forskolin were used, problems with solubility in the assay were encountered. All other materials used were as described previously [9, 19] or were of the highest quality available.

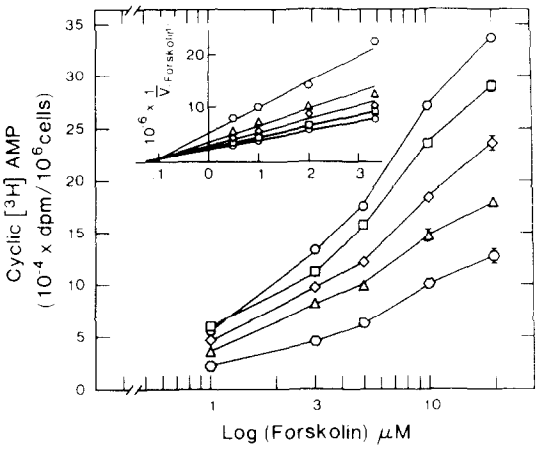

Fig. 1. Effect of ethanol on the concentration–response curve for forskolin-stimulated cyclic [^{3}H]AMP formation in intact murine neuroblastoma cells (clone N1E 115). Intact cells (subculture 9, 20 days after subculture) were prepared for the assay of cyclic [^{3}H]AMP formation as described previously [9]. Cells were then exposed to the indicated concentrations of forskolin with either 0, 100, 300, 500 or 700 mM ethanol present. The assay was terminated after 10 min with TCA. The cyclic [^{3}H]AMP formed was then determined as described [9]. The data shown are representative results from one of four independent experiments. Each point is the mean of triplicates and is the response above basal level (no forskolin present). Inset: double-reciprocal plot of the concentration–response data. The error bars indicate the S.E.M. Symbols: (○) control, (□) 100 mM, (◇) 300 mM, (△) 500 mM and (◯) 700 mM ethanol.

RESULTS

In intact N1E-115 cells prelabeled with [^{3}H]adenine, the addition of forskolin caused a concentration-dependent stimulation of cyclic [^{3}H]AMP formation, which reached a maximum response within 10–15 min. Ethanol did not appear to have any significant effect on the EC_{50} (concentration giving 50% of the maximal response) for forskolin, but did inhibit the response in a concentration-dependent manner (Fig. 1). The EC_{50} values for forskolin in both the absence and the presence of ethanol varied from 8 to 12 μM, which agrees well with that reported by Seamon and Daly (4–25 μM) in various intact cell systems [20]. When the data from the concentration–response curves were plotted as a double-reciprocal plot (inset, Fig. 1), the resultant curves were consistent with a non-competitive type of inhibition. This result was supported by the absence of a significant shift in the EC_{50} for forskolin in the presence of ethanol.

N1E-115 cells have PGE_1 receptors which are coupled to adenylate cyclase [9]. In intact cells prelabeled with [^{3}H]adenine, PGE_1 stimulates the formation of cyclic [^{3}H]AMP, and in the presence of ethanol this response is potentiated in a concentration-dependent manner as previously reported [9]. When forskolin was added to the cells in the presence of PGE_1, the response to PGE_1 was augmented, as has been reported for other agonists in other systems [3].

Figure 2 shows the concentration–response curve for forskolin in the presence of 1 μM PGE_1 and the effect of four different concentrations of ethanol. As expected, ethanol potentiated the PGE_1 response, to a maximum of 200% over control at 500 mM in this case. The augmentation of the effect of PGE_1 by forskolin was inhibited by ethanol, a result shown more clearly in the inset to Fig. 2 where the forskolin-elicited response above PGE_1 alone is plotted. In the presence of 1 μM PGE_1, the EC_{50} for forskolin augmentation was 0.3 μM, and the addition of ethanol did not alter this value significantly. Again, the absence of a shift in the concentration–response curve for forskolin with a reduction in the maximal response is consistent with a non-competitive type of inhibition.

The inhibition of the forskolin response by ethanol was compared between intact N1E-115 cells and a

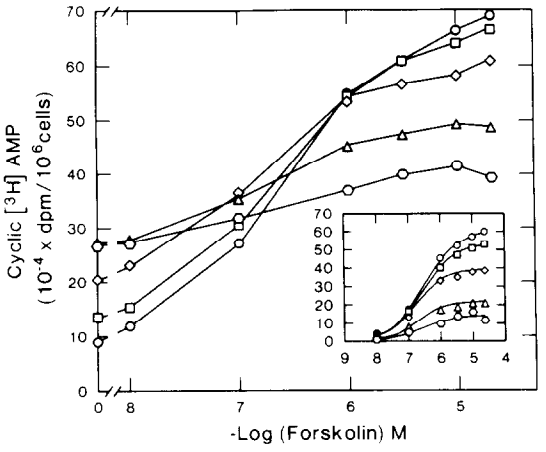

Fig. 2. Effect of ethanol on the concentration–response curve for forskolin-stimulated cyclic [^{3}H]AMP formation in the presence of 1 μM PGE_1 in intact murine neuroblastoma cells (clone N1E-115). Intact cells (subculture 11, 13 days after subculture) were prepared for the assay of cyclic [^{3}H]-AMP formation as described. Cells were then exposed to the indicated concentrations of forskolin and PGE_1 (1 μM) with either 0, 100, 300, 500 or 700 mM ethanol present. The assay was terminated after 10 min with TCA. The cyclic [^{3}H]AMP formed was then determined as described [9]. The data shown are representative results from one of four independent experiments. Each point is the mean of triplicates. Error bars are not shown because they are within the dimensions of the symbols. Symbols: (○) control (no ethanol), (□) 100 mM, (◇) 300 mM, (△) 500 mM and (⬡) 700 mM ethanol. Inset: plot of data showing response above that of PGE_1 alone for indicated ethanol concentrations. (Forskolin + PGE_1 response) − PGE_1 alone = response above PGE_1. Symbols are the same as above.

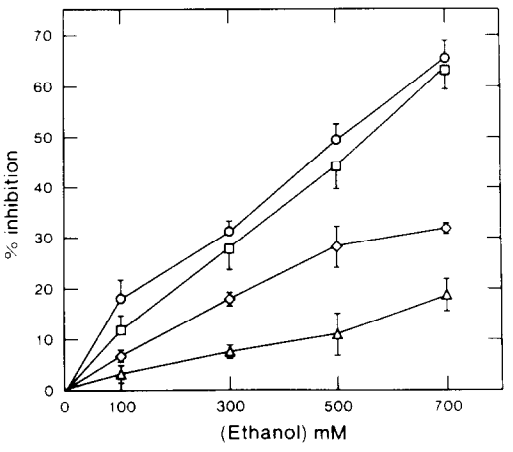

Fig. 3. Comparison of the inhibition by ethanol of forskolin-stimulated adenylate cyclase in intact cells and in membranes. Murine neuroblastoma clone N1E-115 cells (subculture 9–11) were prepared either for the assay of cyclic [^{3}H]AMP formation in intact cells (○, □) or for the assay of adenylate cyclase activity in a membranal preparation (◇, △), as described in the text. The percent inhibition caused by the indicated concentrations of ethanol, of either 1 μM (○, ◇) or 20 μM (□, △) forskolin, is shown. Data are presented as the mean ± S.E.M. and are the combined data from eight independent experiments (four with intact cells, four with membranes) each done in triplicate. Basal values (no forskolin present) were subtracted from forskolin containing samples. Percent inhibition = [(forskolin + ethanol)/forskolin alone] × 100.

membranal preparation at two forskolin concentrations, 1 and 20 μM (Fig. 3). With intact cells there was no significant difference between the percent inhibition caused by ethanol at 1 or 20 μM forskolin, a result suggesting non-competitive inhibition. However, a different effect was observed with the membranal preparation with which there was significantly less inhibition by ethanol at 20 μM forskolin compared to 1 μM forskolin for all ethanol concentrations. This result is more compatible with competitive inhibition. In addition, ethanol was considerably less effective at inhibiting forskolin-stimulated adenylate cyclase in membranes than in intact cells (Fig. 3). We have found a similar phenomenon for the potentiation by ethanol of the PGE_1 response, the effect of ethanol being much less pronounced in membranes than in intact cells [9].

We also reported previously [9] that sucrose and other agents which increase the osmolality of the incubation medium mimic the acute effects of ethanol on intact cells. Therefore, we tested sucrose for its ability to inhibit forskolin-mediated cyclic [^{3}H]-AMP synthesis by intact cells and by membranal preparations from these cells. The data for intact cells (Table 1) show that sucrose in a dose-dependent manner inhibited cyclic [^{3}H]AMP formation mediated by 20 μM forskolin. The magnitude of the inhibition was similar to that found with ethanol and was no different when 1 μM forskolin was used (data not shown). In addition, in two independent experiments with membranal preparations from these cells, 200 mM sucrose inhibited the effects of 20 μM forskolin by only 14.8 and 8.2% indicating that, like ethanol, sucrose was more effective as an inhibitor with intact cells.

Table 1. Effect of sucrose on forskolin-mediated cyclic [^{3}H]AMP synthesis by murine neuroblastoma cells*

Conc of sucrose (mM)	Cyclic [^{3}H]AMP response (dpm ± S.E.M.)	% Inhibition
	10^{-2} × dpm/10^6 cells	
0	1850 ± 30	
50	1580 ± 40	14.7
100	1400 ± 100	24.4
200	1189 ± 3	35.8
300	1173 ± 1	36.7

* Intact cells (subculture 11, 13 days after subculture) were prepared for the assay of cyclic [^{3}H]AMP formation as described. Cells were then exposed to 20 μM forskolin and the indicated concentrations of sucrose. The assay was terminated after 10 min with TCA. The cyclic [^{3}H]AMP formed was then determined as described [9]. The data shown are representative results from one of two independent experiments, each determined in triplicate. Basal levels of cyclic [^{3}H]AMP which averaged 6100 ± 300 dpm/10^6 cells were subtracted from the original data. All results with sucrose were significantly different from no sucrose with $P < 0.0005$.

DISCUSSION

With a few exceptions the results presented here confirm those reported previously by Huang *et al.* [16] and Robberecht *et al.* [17] concerning the inhibition of forskolin-stimulated adenylate cyclase by ethanol. Both of the reported studies used membranal preparations to assess the effect of ethanol and both showed a concentration-dependent inhibition, but the type of inhibition differed somewhat between these studies. Huang *et al.* reported that in bovine corpora lutea the inhibition was a competitive type, while in rat cardiac membrane Robberecht *et al.* found a mixed type of inhibition by ethanol. Our study primarily assessed ethanol's inhibition of the forskolin response in intact N1E-115 cells. The type of inhibition that we observed appeared to be non-competitive in nature because there was no shift to the right in the forskolin concentration–response curve in the presence of ethanol and there was no significant difference in the amount of inhibition caused by ethanol in the presence of either 1.0 or 20.0 μM forskolin (Fig. 3). Forskolin potentiated the effect of PGE_1 on cyclic AMP synthesis and had a greatly decreased EC_{50} in the presence of 1 μM PGE_1 (from 8–12 μM to 0.3 μM). The addition of ethanol to the forskolin concentration–response curve in the presence of PGE_1 caused an inhibition of forskolin's potentiation of the PGE_1 response, with no effect on the forskolin EC_{50}, a result which again is consistent with non-competitive inhibition.

However, when we determined the effect of ethanol in a membranal preparation, a different type of inhibition was observed. The results from Fig. 3 suggest that with membranes the inhibition is more of a competitive nature, since the amount of inhibition was less in the presence of a greater concentration of forskolin. This finding agrees more closely with those previously reported [16, 17]. The results reported here show not only that the nature of ethanol's inhibition is different in intact cells versus membranes but also that the magnitude of the inhibition is different. As shown in Fig. 3, there was an inhibition of about 60% of forskolin-cyclic AMP synthesis at 700 mM ethanol in intact cells while in membranes there was at best an inhibition of 30%. A similar difference is seen when one compares the potentiation of the PGE_1-cyclic AMP response by ethanol. Thus, in intact cells 200 mM ethanol causes an increase of 148% over control while in membranes it causes a maximim of 15–20% increase [9]. Harper and Brooker [21] found similar results when they studied the potentiation of isoproterenol-stimulated cyclic AMP formation in rat parotid gland by alcohols. In addition, we found that sucrose mimicked the effects of ethanol in this study (Table 1) and in our previous report [9]. All these findings raise the question of whether some of the pharmacological effects of ethanol are related to its non-specific effect on cellular osmolality rather than to a more specific effect of this agent.

Nonetheless, ethanol has been shown to affect the fluidity of cellular membranes. Upon acute exposure to ethanol, this fluidity increases and with chronic exposure the lipid composition of the membrane becomes modified to form a more rigid or ordered state [12, 22–24]. These results suggest that, in the intact cell, the entire plasma membrane acts as a unit in response to the extracellular environment. Our data and those of Harper and Brooker [21] suggest that homogenization of the cell, which causes the destruction of the unit membrane, results in a significant alteration in the way the membrane can respond to perturbations that affect the adenylate cyclase system.

The mechanism by which ethanol inhibits forskolin-mediated cyclic AMP synthesis is uncertain. Ethanol could be directly inhibiting the binding of forskolin to its activation site. The recent availability of [^{3}H]forskolin gives the opportunity to study the effects of ethanol on the direct binding of forskolin. Another possible locus for the effect of ethanol could be at a site between forskolin's binding and its activation of the catalytic unit of adenylate cyclase. If forskolin were to act through some other regulatory protein before it activates the catalytic unit, as suggested [4, 5], then ethanol could disrupt this interaction through its alteration of membrane fluidity or by directly affecting this protein. Further investigations of the interaction of forskolin and ethanol are warranted and could possibly lead to a better understanding of the adenylate cyclase system and the sites of action of ethanol and forskolin.

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