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STIMULATION OF RAT C6 GLIOMA ECTO-5′-NUCLEOTIDASE BY CHRONIC ETHANOL TREATMENT

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Abstract—Treatment of rat C6 glioma cells in culture with 100 mM ethanol led to increased ecto-5′-nucleotidase activity. Studies were performed to obtain information on the mechanism(s) of action for this effect. Growth of C6 glioma cells in 100 mM ethanol for 6 days increased ecto-5′-nucleotidase activity but had no effect on the pH optima or substrate specificity of the ecto-enzyme. A comparison of enzyme activity of intact cells with that of disrupted cells revealed a selective stimulation of the ecto-enzyme, i.e. the enzyme on the outer plasma membrane. Kinetic studies of cells continuously treated with 100 mM ethanol for 6 days showed an apparent increase in affinity of the ecto-enzyme for 5′-AMP, with no appreciable change in the maximum velocity (V_{max}). The concentration-dependent inhibition of ecto-5′-nucleotidase activity by Concanavalin A (Con A) was antagonized by ethanol treatment. Double reciprocal plot analysis showed that ethanol acted in an apparently noncompetitive manner with respect to Con A inhibition. It appears that the availability of the catalytic site of the ecto-5′-nucleotidase to exogenous substrates was increased. The data are consistent with rearrangement of the plasma membrane resulting from continuous exposure to ethanol.

The enzyme 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) is a component of most mammalian cell plasma membranes and is often used as an enzyme marker in studies on this cellular structure [1]. In several cell types this enzyme has been shown to behave as an ecto-enzyme, that is, as one being present on the outer surface of the cell membrane [2–5].

In the nervous system, 5′-nucleotidase is associated with glial cell membranes [6, 7] and has been shown to be an ecto-enzyme in glioma [5] cell cultures. The enzyme appears to be part of an extracellular chain of enzymes that catalyze the breakdown of membrane-impermeable nucleotides into membrane-permeable nucleosides [8]. In nervous tissue the enzyme may be particularly important for the production of adenosine, a nucleoside with considerable neuromodulating activity [9–12].

We previously reported an increase in the ecto-5′-nucleotidase activity of the rat glioma C6 cell line after exposure to ethanol [12]. In that study we determined that the stimulatory effect of ethanol treatment was not a consequence of changes in cell viability, of increased leakiness of substrate into or enzyme out of the cell, or of the acute effect of ethanol treatment. In the present investigation we have examined enzyme characteristics of the ecto-5′-nucleotidase in order to elucidate possible mechanisms involved in the ethanol-induced enzyme stimulation. Comparisons have been made between enzyme activity in cell homogenates and intact cells, with and without prior treatment with 100 mM ethanol. Substrate specificity, the effect of pH on enzyme activity, enzyme kinetics and inhibition of activity by Concanavalin A were also studied.

METHODS

Cell culture. Cells were grown in a medium consisting of 90% Dulbecco's modified Eagle's medium and 10% fetal calf serum (v/v), supplemented with penicillin G (50 units/ml) and streptomycin sulfate (25 μg/ml). Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°. The medium in the culture flasks (15 ml) was changed every 2 days for 5–6 days at which time the cells were detached from the flasks by treatment with trypsin and suspended in fresh growth medium. One-milliliter aliquots of the culture (1–4 × 10^4 cells/ml) were transferred into 60 mm diameter dishes to which 4 ml of growth medium was added. Every second day as well as 18–24 hr prior to use in the present experiments, the cultures were replenished with fresh medium.

Cell cultures chronically§ exposed to ethanol received the drug (100 mM initial concentration) in the growth medium and were otherwise treated in the same manner as control cultures. Direct measurement revealed evaporation of the ethanol

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§ In the context of this study, chronic exposure to ethanol is equated with continuous treatment, as described in the Methods. The two phrases will be used interchangeably.

to 50–60 per cent of the initial concentration during the 48 hr between media changes.* Therefore, continuously treated cells were actually exposed to less than 100 mM ethanol for most of the treatment period. For clarity, however, reference to ethanol concentrations will be to the initial concentrations.

Chronic treatment of C6 cells with 100 mM ethanol has only a slight and variable effect on cell growth.† The effect of continuous treatment with 100 mM ethanol on 5′-nucleotidase as a function of cell growth has been examined [12]. With chronic stimulation, activity increased with cell density but was not dependent upon it. In the studies reported here, the cell densities of the control cultures and of the chronically treated cultures were not significantly different.

For the studies on the acute effects of ethanol, cultures were grown as above. Acute exposure occurred with the addition of ethanol-containing incubation medium to cells rinsed three times with incubation medium (see below). A 5-min equilibrium period was allowed to elapse prior to measurement of enzyme activity.

Assays for 5′-nucleotidase. The measurement of the ecto-5′-nucleotidase activity of intact C6 cell cultures growing in 60 mm dishes was as described previously [5, 12]. Incubation times varied but were maintained within the linear range for activity of the enzyme.

Total cellular 5′-nucleotidase activity was measured in cells collected by removing the growth medium and washing the cell monolayer three times with 10 ml of fresh incubation medium (130 mM NaCl, 1 mM $MgCl_2$ and 5.5 mM glucose in 40 mM Tris–HCl buffer, pH 7.4). Cells were removed from the surface of the dish by scraping with a rubber policeman. The suspended cells were collected by centrifugation and frozen at −20° until assayed. Cells were lysed in 1 ml of doubly distilled water using a Potter–Elvehjem homogenizer fitted with a teflon pestle. Fifty-microliter aliquots of homogenate were diluted with incubation medium and assayed at 37° with the specified substrate in a total volume of 600 μl; incubations were performed in duplicate. After 20 min, the reaction was terminated by the addition of chilled trichloroacetic acid to give a final concentration of 5% (w/v). The samples were centrifuged and the liberated inorganic phosphate was measured in the supernatant fluid by the method of Gomori [13]. Values were corrected for non-enzymatic hydrolysis of substrate and for endogenous inorganic phosphate in the homogenates.

Materials. The tissue culture medium, serum and antibiotics were purchased from Gibco (Grand Island, NY). Plastic culture ware was obtained from Falcon Plastics (Oxnard, CA). Rat glioma cell line C6 (CCL 106) was obtained from the American Types Culture Collection (Rockville, MD). Concanavalin A, grade IV, was obtained from the Sigma Chemical Co. (St. Louis, MO) and used without further purification. Phosphate ester-containing compounds and α-methyl-D-mannoside were also obtained from Sigma. Ethanol (95%, v/v) (U.S.P. equivalent) was obtained as an azeotrope with water and used without further purification.

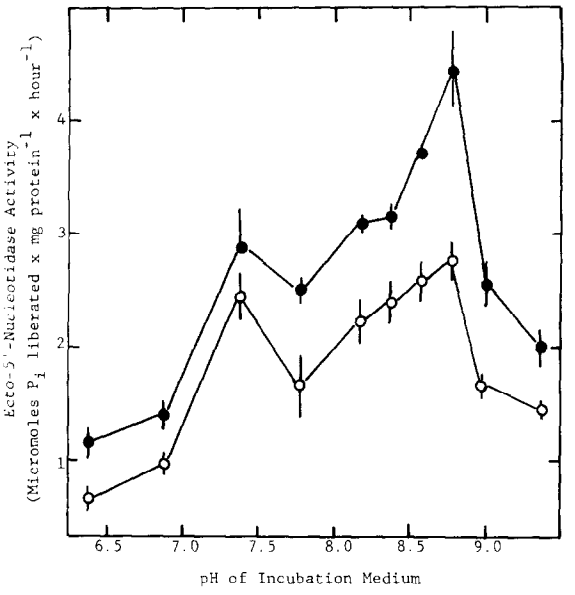

Fig. 1. Effect of pH on ecto-5′-nucleotidase activity of control and chronically ethanol-treated cells. Cultures were grown for 6 days in the presence or absence of 100 mM ethanol in the growth medium. Forty mM Tris–HCl buffer was used throughout and adjusted with either 1 N HCl or solid Tris to the desired pH. Open circles (○) are controls; closed circles (●) are chronically ethanol-treated cells. Incubation time was 6 min. Each point is the mean of three determinations. Vertical bars represent ± S.E.

RESULTS

Chronic ethanol treatment and the pH optimum of ecto-5′-nucleotidase. The activity of ecto-5′-nucleotidase of chronically ethanol-treated and nontreated C6 cells was determined between pH 6.5 and 9.5 (Fig. 1). Nonspecific ecto-phosphatase activity [4] was measured at the same time at each pH and subtracted from the total activity to give specific ecto-5′-nucleotidase activity [12]. C6 cells were found to have a two pH optima for ecto-5′-nucleotidase activity, at pH 7.4 and 9.0. Chronic treatment with ethanol did not change the pH optima; however, treatment resulted in a more active enzyme near the higher of the pH optima.

Substrate specificity. Ecto-enzymatic activity towards different phosphate ester-containing substrates was examined in treated and nontreated cells. Total enzyme activity in whole cell homogenates using the same substrates was also measured. The results are shown in Table 1. The ecto-enzyme hydrolyzed 5′-AMP most rapidly, but was substantially active with other 5′-mononucleotides as substrate. The ecto-enzyme showed low activity with *p*-nitrophenylphosphate and glucose-6-phosphate (Table 1) as well as with 5′-dAMP. The relative order of activity was found to be 5′-AMP>5′-

* P. J. Syapin, unpublished data.
† P. J. Syapin, Ph.D. thesis.

Table 1. Effect of chronic ethanol treatment on substrate specificity of ecto- and total 5′-nucleotidase activity of C6 cells*

Substrate [3 mM]	Ecto-5′-nucleotidase activity (μmoles P_i formed·mg protein^{-1}·hr^{-1})			Total 5′-nucleotidase activity (μmoles P_i formed·mg protein^{-1}·hr^{-1})		
	Control cells (A)	Ethanol-treated cells (B)	(B − A)	Control cells (C)	Ethanol-treated cells (D)	(D − C)
5′-AMP	1.70 ± 0.10	2.63 ± 0.10	0.93	3.13 ± 0.16	4.17 ± 0.17	1.04
5′-CMP	1.29 ± 0.09	1.64 ± 0.08	0.35	2.34 ± 0.10	2.59 ± 0.28	0.25
5′-GMP	0.79 ± 0.01	1.03 ± 0.03	0.27	1.23 ± 0.23	1.46 ± 0.83	0.23
5′-IMP	0.77 ± 0.01	1.11 ± 0.03	0.34	1.33 ± 0.13	1.70 ± 0.20	0.37
5′-dAMP	0.31 ± 0.05	0.33 ± 0.12	0.02	1.02 ± 0.10	2.24 ± 0.15	1.22
p-Nitrophenylphosphate	0.19 ± 0.01	0.21 ± 0.02	0.02	0.18 ± 0.05	0.71 ± 0.07	0.53
Glucose-6-phosphate	0.05 ± 0.01	0.09 ± 0.01	0.04	ND†	ND	

* Cells were grown in the presence or absence of 100 mM ethanol in the medium for 6 days. Values are mean ± S.E. of three determinations for each substrate. Enzyme activity was determined on intact cells (ecto-activity) and cell homogenates (total activity) as described in Methods.

† ND = not determined.

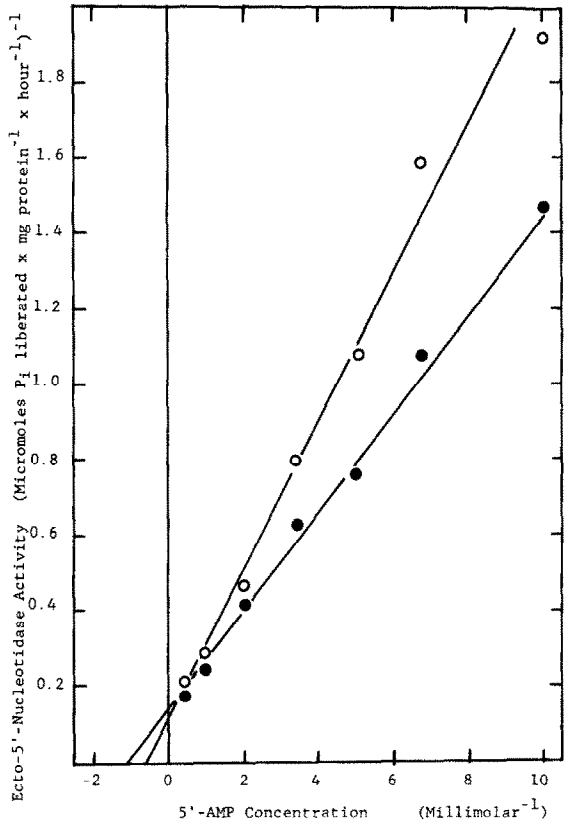

Fig. 2. Lineweaver–Burk plots for control and chronically ethanol-treated cells. Cultures were grown for 6 days in the presence or absence of 100 mM ethanol in the growth medium. Substrate concentrations varied as indicated, with the *p*-nitrophenylphosphate concentration remaining at 1 mM. Open circles (○) are controls; closed circles (●) are chronically ethanol-treated cells. Initial velocity was measured as μmoles P_i liberated·mg protein^{-1}·hr^{-1}. Incubation time was 8 min. Each point is the mean of three determinations. Lines were constructed by the method of least squares.

CMP > 5′-GMP = 5′-IMP for control cells and remained unchanged after chronic treatment of cells with 100 mM ethanol. The greatest per cent stimulation by ethanol treatment was seen using 5′-AMP as substrate. The difference in activity between control and treated cells (Table 1, column B − A) was 2.7 times greater using 5′-AMP as substrate than using 5′-CMP, the second most active compound, as substrate.

The specificity of total 5′-nucleotidase toward these substrates is also shown in Table 1. The relative order of activity for control and treated cell homogenates was again 5′-AMP > 5′-CMP > 5′-GMP = 5′-IMP. Cell homogenates contained three to six times the activity of intact cells toward 5′-dAMP. Whether this was due to 5′-nucleotidase or another enzyme was not determined.

From the data in Table 1, ecto-5′-nucleotidase activity of control cells was found to be 59 ± 2 per cent of the total cellular 5′-nucleotidase activity. After chronic ethanol treatment this value was increased to 66 ± 2 per cent ($P < 0.05$, Student's *t*-test for paired data). The increase in 5′-nucleotidase activity following chronic ethanol treatment was calculated for each substrate and is shown in columns (B − A) and (D − C) of Table 1 for intact and homogenized cells, respectively. This calculation shows that exposure to ethanol did not significantly increase the enzyme activity of cell homogenates compared to that of intact cells. The data indicate that chronic treatment with 100 mM ethanol stimulated only the 5′-nucleotidase present as the ecto-enzyme.

Enzyme kinetics. Lineweaver–Burk plots were constructed for the ecto-5′-nucleotidase of non-ethanol and chronically ethanol-treated intact cells (Fig. 2). The K_m values were determined after least squares regression analysis of the data. A decrease in the K_m (increased affinity) from 1.67×10^{-3} M for the control cells to 1.00×10^{-3} M for the cells exposed to 100 mM ethanol was observed. The V_{max} increased only slightly after ethanol treatment.

Studies of inhibition by Concanavalin A (Con A).

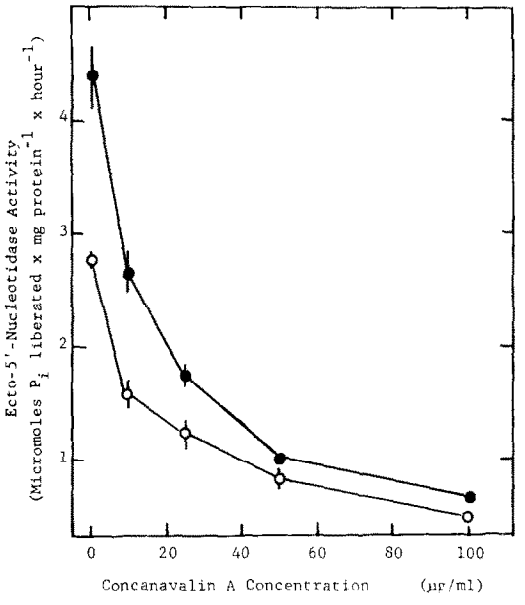

Fig. 3. Inhibition of ecto-5′-nucleotidase activity by Concanavalin A. Cultures were grown for 6 days in the presence or absence of 100 mM ethanol in the growth medium. Ecto-5′-nucleotidase activity was assayed in the incubation medium minus glucose and containing the indicated concentration of Concanavalin A. Cultures were preincubated for 5 min at 37° in the presence of Concanavalin A prior to determination of activity. Incubation time was 10 min. Open circles (○) are controls; closed circles (●) are chronically ethanol-treated cells. Each point is the mean of three determinations. Vertical bars denote ± S.E.

Con A is a potent inhibitor of ecto-5′-nucleotidase activity in intact C6 cells [15]. Inhibition is due to the binding of the lectin to the cell surface. In the present study, Con A was found to inhibit the ecto-5′-nucleotidase activity of cells chronically exposed to 100 mM ethanol and of nonexposed cells in the same manner (Fig. 3).

A double reciprocal plot of the inhibition by Con A of exposed and nonexposed cells revealed that ethanol treatment acted as an apparently noncompetitive antagonist to Con A inhibition (Fig. 4). Similar results were found after acute treatment of cells with 100 mM ethanol (Fig. 4). The data in Table 2 confirm that the Con A inhibited the ecto-5′-nucleotidase activity by binding to the cell. Enzyme activity was reduced in cells that were exposed to Con A and subsequently rinsed to remove unbound Con A from the medium. The inhibition was also reversed by treatment with α-methyl-D-mannoside, which competes for the Con A binding site [14]. Cells treated with Con A and then washed were not stimulated acutely by 100 mM ethanol, but when Con A was competitively removed with α-methyl-D-mannoside, acute treatment with ethanol stimulated enzyme activity. Prior exposure of cells to 100 mM ethanol for 6 days made them unresponsive to the acute stimulatory action of ethanol seen with control cells (Table 2). This form of tolerance has been described in detail [12] and appears to be retained even after successive treatment with Con A, α-methyl-D-mannoside and acute ethanol.

DISCUSSION

The data presented in this study support the hypothesis that enhanced 5′-nucleotidase activity of intact cells chronically exposed to ethanol occurs through changes in the plasma membrane. This

Table 2. Concanavalin A inhibition of ecto-5′-nucleotidase activity—effect of carbohydrate reversal and acute ethanol on chronically ethanol-treated and non-treated cells*

Treatment	Ecto-5′-nucleotidase activity (%) Non-ethanol-treated cells	Chronically ethanol-treated cells
None	100†	100‡
Ethanol (100 mM)	148	109
Con A (10 μg/ml)	70	70
Con A (10 μg/ml) then washed 3×	70	79
Con A (10 μg/ml) washed 3× + ethanol (100 mM)	71	74
Con A (10 μg/ml) washed 3× + α-methyl-D-mannoside (0.26 m)	113	113
Con A (10 μg/ml) washed 3× + α-methyl-D-mannoside (0.26 M) + ethanol (100 mM)	139	110

* Cells were grown in the presence or absence of 100 mM ethanol for 6 days. Treatment was carried out at 37° by incubating cultures in 5 ml of the following: Con A in medium minus glucose, α-methyl-D-mannoside in medium minus glucose or ethanol-containing medium. Rinses were made with normal incubation medium. Treatment times were 5 min with Con A, 10 min with α-methyl-D-mannoside and 5 min with acute ethanol. Activity was measured after appropriate treatment by an 8-min incubation. Each entry was calculated based upon the mean value of three replicate determinations for each condition.

† Activity = 1.54 ± 0.29 μmoles P_i liberated·mg protein^{-1}·hr^{-1}.

‡ Activity = 2.69 ± 0.35 μmoles P_i liberated·mg protein^{-1}·hr^{-1}.

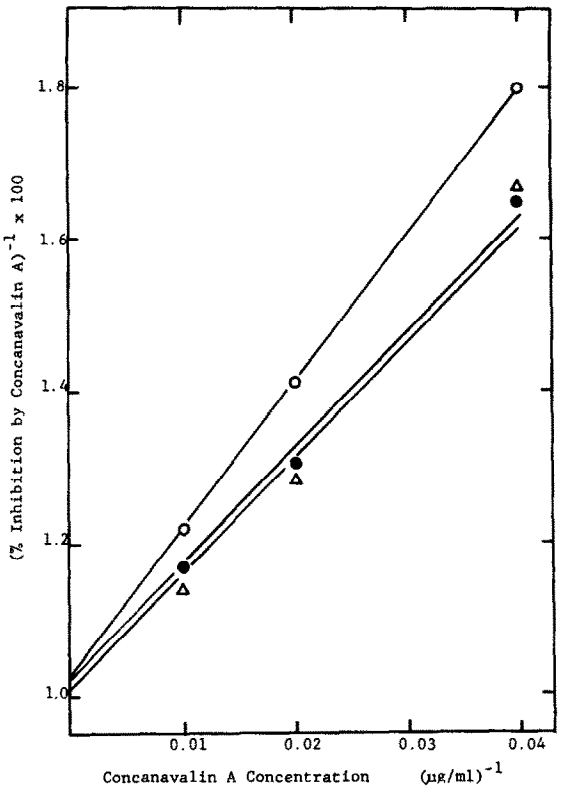

Fig. 4. Double reciprocal plots of inhibition of ecto-5′-nucleotidase activity by Concanavalin A and the effect of ethanol treatment. Cultures were or were not exposed to 100 mM ethanol for 6 days. Cells acutely treated with 100 mM ethanol were also grown for 6 days. Cultures were preincubated for 5 min with Concanavalin A in the incubation medium minus glucose at the concentrations indicated. Incubation time was 10 min. Open circles (○) show control cell activity; closed circles (●) show chronically exposed cell activity; and open triangles (△) show acutely exposed cell activity. Each point is the mean of three determinations. Best-fit lines were constructed by the method of least squares regression.

interpretation is based upon the following observations. First, 5′-nucleotidase activity of intact cells resulted from the breakdown of substrate at the external cell surface; thus an ecto-enzyme is involved. This finding was described previously [5, 12]. Second, ethanol treatment antagonized Con A inhibition of ecto-5′-nucleotidase. Con A, which binds to specific sugars at the cell surface [14], powerfully inhibited the enzyme activity of intact C6 cells, possibly through a reduction in the amount of active enzyme available on the cell surface [15]. Since Con A binding reduced the activity of chronically treated cells to control levels, the increased activity seen after chronic ethanol treatment presumably was due to enzymatic activity expressed at the cell surface. The apparently noncompetitive nature of ethanol's antagonism of the Con A effect (Fig. 4) suggests that the ethanol was acting at sites in the membrane different from those which bind Con A. Third, studies on both intact and disrupted cells with various 5′-mononucleotides showed that the increased 5′-nucleotidase activity resulted from a selective increase in ecto-5′-nucleotidase activity (Table 1). Fourth, the K_m of intact cells after chronic ethanol exposure (Fig. 2) was reduced without changing the V_{max} appreciably. This result suggests that there is an increased affinity for the substrate by the enzyme and/or an increased exposure of the catalytic site on the enzyme [16]. Fifth, previous investigation has shown that cell viability, measured by dye staining, and leakage of cytoplasmic enzymes into the media are not altered by exposure of intact cells to 100 mM ethanol [12].

In the liver, ethanol increases enzyme activity through enzyme induction, as seen for drug-metabolizing enzymes [17]. However, ethanol appears not to have induced 5′-nucleotidase in the C6 cell line. 5′-Nucleotidase was present both intracellularly and as the ecto-enzyme in C6 cells, as demonstrated by increased activity in homogenized versus intact cell preparations (see Table 1). If, therefore, enzyme induction had occurred, it would be expected to have led to an increase in activity in cell homogenates that was greater than in intact cells. This, however, did not occur. Instead, the increased activity measured in cell homogenates can be accounted for solely by the increased activity measured at the cell surface. The possibility that ethanol might have selectively induced only ecto-5′-nucleotidase of C6 cells is also unlikely. If induction of only the ecto-enzyme occurred, the V_{max} of the system would be expected to increase. This was not observed.

A third point which argues against enzyme induction is the antagonism by ethanol treatment of inhibition by Con A (Fig. 4). If chronic ethanol exposure served merely to increase the number of enzyme molecules, a competitive antagonism should be observed. The data, however, correspond to the type of curves found for a noncompetitive antagonism [18].

These findings strongly support the idea that chronic treatment of C6 cells with ethanol stimulates ecto-5′-nucleotidase activity through a change in plasma membrane conformation [12]. The exact components or primary mechanism ultimately responsible still remains to be determined. Whether the 5′-nucleotidase molecule itself or its surrounding physicochemical environment is altered will require further studies.

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