

Ethanol alters the adenosine receptor- N_i -mediated adenylate cyclase inhibitory response in rat brain cortex in vitro

F. Bauché, A.M. Bourdeaux-Jaubert, Y. Giudicelli and R. Nordmann

Department of Biomedical Research on Alcoholism and Department of Biochemistry, Faculty of Medicine Paris-West, 45 rue des Saints-Pères, 75006 Paris, France

Received 27 May 1987

It has been suggested that ethanol stimulates adenylate cyclase in vitro through an increased function of N_s , the activatory component of adenylate cyclase. Because of the interaction of N_s with N_i , the adenylate cyclase inhibitory component, we have studied the effect of ethanol (0.05–0.2 M) on N_i -mediated adenylate cyclase inhibition caused by the adenosine analog N^6 -phenylisopropyladenosine (N^6 -PIA) in brain cortical membranes. Ethanol did not alter N^6 -PIA binding to the adenosine R_i -receptors, stimulated slightly basal adenylate cyclase activity but abolished adenylate cyclase inhibition due to N^6 -PIA, suggesting an effect of ethanol on the inhibitory coupling pathway. This was further supported by loss of the adenylate cyclase inhibitory response to GTP ($> 10^{-5}$ M). It thus seems that, besides its effect on the N_s system, ethanol may also impair N_i -mediated adenylate cyclase responses in rat cerebral cortex.

Synaptosome; Adenylate cyclase; Enzyme inhibition; Adenosine; Ethanol effect

1. INTRODUCTION

In almost all tissues, adenylate cyclase activity is regulated by two opposite systems. These systems consist of activatory and inhibitory receptors that are coupled to the adenylate cyclase catalytic subunit (C) via two different heterotrimeric proteins called N_s and N_i . Binding of stimulatory and inhibitory agonists to their respective receptors initiates a GTP-dependent dissociation of N_s and N_i into their α - and $\beta\gamma$ -subunits [1,2]. The role of the $\beta\gamma$ -subunit, which is common to N_s and N_i , is to sequester and inactivate the α -subunits. Dissociation of N_s releases the α_s -subunit which, when bound to GTP, directly activates C. Dissociation

of N_i releases the α_i -subunit together with $\beta\gamma$ which, in turn, reassociates with α_s -GDP and thereby leads to deactivation of C [2–4].

Several authors [5–7] have shown that ethanol in vitro increases basal as well as neurotransmitter-stimulated adenylate cyclase activities in the central nervous system. From these studies it was concluded that activation of both the catalytic subunit and the N_s regulatory protein of adenylate cyclase was the molecular mechanism responsible for this effect of ethanol. Because of the dual regulation of adenylate cyclase, it cannot be excluded, however, that decreased activity of the inhibitory coupling system may also be accounted for by the stimulatory effect of ethanol on adenylate cyclase in the central nervous system. The aim of the present study was to test this hypothesis by investigating the effects of ethanol in vitro on the inhibitory response of brain cortex adenylate cyclase to the adenosine R_i analog, N^6 -phenylisopropyladenosine (N^6 -PIA).

Correspondence address: Y. Giudicelli, Department of Biomedical Research on Alcoholism and Department of Biochemistry, Faculty of Medicine Paris-Ouest, 45 rue des Saints-Pères, 75006 Paris, France

2. MATERIALS AND METHODS

2.1. Preparation of crude brain membranes

Fed male Wistar rats (160 ± 20 g) were killed by decapitation and their brains quickly removed. Cortexes were dissected into small pieces, washed and homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM MgCl_2 . Homogenates were centrifuged ($600 \times g$ for 10 min at 4°C), and the supernatant collected and recentrifuged at $15000 \times g$ for 10 min at 4°C . The resulting pellet was resuspended in Tris-HCl buffer at a final protein concentration of about 1 mg/ml and was immediately used for adenylate cyclase assays. For N^6 -PIA binding studies, membranes were prepared in a similar way except that the $15000 \times g$ pellet was additionally incubated for 20 min at 37°C with adenosine deaminase (60 mU/mg protein), recentrifuged ($15000 \times g$ for 10 min at 4°C), resuspended (1 mg/ml) and stored in liquid nitrogen prior to use.

2.2. Adenylate cyclase assays

After exposure to various ethanol concentrations for 5 min at 0°C , fresh membranes (10 μg protein) were incubated in 0.3 mM ATP, 30 mM Tris-HCl (pH 7.4), 80 mM NaCl, 5 mM creatine phosphate, 25 U/ml creatine kinase, 25 U/ml adenosine deaminase, 0.04% bovine serum albumin, 0.5 mM Ro 20-1724 and, unless otherwise stated, 5 mM MgCl_2 and 4 μM GTP, in a final volume of 0.1 ml, for 15 min at 25°C . Reactions were stopped by dilution with 1 ml ice-cold buffer (50 mM Tris-HCl, 4 mM EDTA) followed by boiling at 100°C for 3 min. Tubes were centrifuged and the cyclic AMP produced was assayed in an aliquot of the supernatant as described [8]. In some experiments, the adenylate cyclase assay medium used was the same as that above except for the GTP and MgCl_2 concentrations which were 20 μM and 2 mM, respectively.

2.3. N^6 -PIA-binding assays

Thawed membranes were preincubated in the absence or presence of ethanol as described above and then assayed for N^6 -PIA binding as follows: membranes (300 μg protein) were incubated with N^6 - $[\text{}^3\text{H}]\text{PIA}$ (over the range 0.5–10 nM) in a total volume of 500 μl of 50 mM Tris-HCl buffer (pH 7.4) for 20 min at 37°C . Incubations were ter-

minated by adding 4 ml cold buffer followed by rapid vacuum filtration on Whatman GF/B glass-fiber filters. Filters were rapidly washed with 2×4 ml ice-cold buffer, and dried and counted in 10 ml PCS (Amersham) with a counting efficiency of 40%. Non-specific binding determined in the presence of 10 μM N^6 -PIA at each level of ligand concentration was unchanged by ethanol preincubation and averaged 6% of total binding at 10 nM N^6 - $[\text{}^3\text{H}]\text{PIA}$. All binding values reported refer to specific binding.

2.4. Materials

ATP, creatine phosphate, creatine kinase, GTP, adenosine deaminase and bovine serum albumin (fraction V, fatty acid-poor) were from Sigma, N^6 -PIA from Boehringer Mannheim and N^6 - $[\text{}^3\text{H}]\text{PIA}$, cyclic $[\text{}^3\text{H}]\text{AMP}$ and cyclic AMP-binding protein from Amersham. Ro 20-1724 was kindly provided by Hoffman-La Roche.

3. RESULTS

Basal adenylate cyclase activity (studied in the presence of 4 μM GTP, 5 mM Mg^{2+} , 80 mM Na^+) in control membranes was 201 ± 25 pmol cyclic AMP/min per mg protein. In the presence of 100 nM N^6 -PIA, this activity decreased by $15 \pm 1.5\%$ ($0.001 < P < 0.01$). Under the same experimental conditions, basal activity was not significantly affected by preincubation with ethanol concentrations up to 0.1 M, but was significantly increased ($+17 \pm 4\%$, $n = 8$, $P < 0.001$) by preincubation with 0.2 M ethanol. In contrast and under the same incubation conditions, preincubation with ethanol (0.1 or 0.2 M) completely abolished the adenylate cyclase inhibitory response to 100 nM N^6 -PIA (fig.1).

To investigate whether the latter effect of ethanol could be linked to altered adenosine receptors, we have compared the characteristics of N^6 - $[\text{}^3\text{H}]\text{PIA}$ binding to these receptors in membranes preincubated with or without ethanol. As shown in table 1, neither the receptor-dissociation constant (K_D) nor their number (B_{max}) was significantly affected by preincubation with 0.1 M ethanol. These results, which exclude altered adenosine-receptor binding as the cause of the ethanol-induced loss of adenylate cyclase response to N^6 -PIA, rather suggest an effect of ethanol on

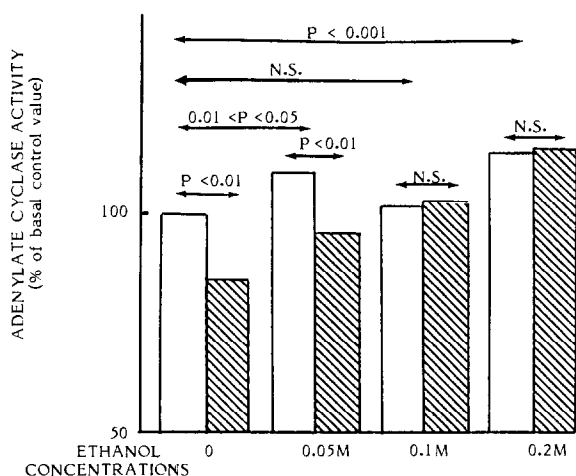


Fig.1. Effects of ethanol on the adenylyl cyclase inhibition caused by N^6 -PIA. Adenylyl cyclase activity was determined in the absence (open bars) or presence of 100 nM N^6 -PIA (hatched bars) after preincubation (5 min at 0°C) of the membranes with various ethanol concentrations. Each value is expressed as a percentage of the activity found in membranes not exposed to ethanol and N^6 -PIA (201 ± 25 pmol cAMP/min per mg protein) and represents the mean of 8 experiments. Statistical significances were analyzed by paired Student's *t*-test.

the GTP-dependent N_i -mediated inhibitory transducing system.

To test this hypothesis, we compared the adenylyl cyclase dose-response curves to GTP (0.1–100 μ M) in membranes preincubated in the absence (control) or presence of 0.1 M ethanol (fig.2). In agreement with previous studies [9,10], the curves obtained in control membranes showed a biphasic response with, at GTP concentrations up to 4 μ M, an activation, and, at higher concentrations, a progressive reversal of this activation. In contrast, membranes preincubated with 0.1 M ethanol, although eliciting basal values (absence of GTP) and stimulatory responses similar to those found in control membranes, failed to show the reversion phase which was observed at high GTP concentrations in control membranes (fig.2). These results demonstrate that, under the present experimental conditions, ethanol suppresses the adenylyl cyclase inhibitory responses to both N^6 -PIA and GTP.

Finally, we investigated the influence of ethanol on the adenylyl cyclase inhibitory response to

Table 1

Effects of ethanol on the adenosine R_i receptor binding parameters

	Dissociation constant (K_D) (nM)	Maximal number (B_{max}) (fmol/mg protein)
Control	1.26 ± 0.31 (6)	700 ± 56 (6)
Ethanol (0.1 mM)	1.54 ± 0.38 (6) $P > 0.05$	721 ± 71 (6) $P > 0.05$

After preincubation for 5 min at 0°C in the absence or presence of 0.1 M ethanol, rat brain cortical membranes were exposed to various concentrations of N^6 -PIA for 20 min at 37°C. Non-specific binding, B_{max} and K_D values were determined as described in section 2. Each value represents the mean \pm SE of the data from 6 separate experiments performed in triplicate. Statistical significances were analyzed by paired Student's *t*-test.

N^6 -PIA in a medium containing higher GTP (20 μ M) and lower $MgCl_2$ (2 mM) concentrations. Under these conditions, the inhibitory potency of

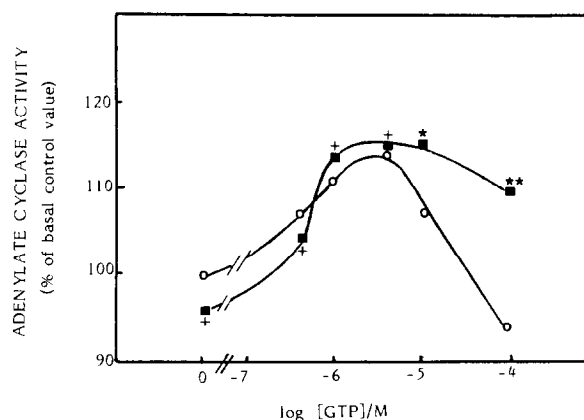


Fig.2. Influence of ethanol on the biphasic modulation of adenylyl cyclase by GTP. Rat brain cortical membranes were preincubated for 5 min at 0°C without (○) or with (■) 0.1 M ethanol and assayed for adenylyl cyclase activity at various concentrations of GTP as described in section 2. Each value is expressed as the percentage of basal control activity, in the absence of ethanol and guanine nucleotide (222 ± 27 pmol cAMP/min per mg protein) and is the mean of 3 experiments. Statistical significances were analyzed by paired Student's *t*-test with: + non significant; * $0.01 < P < 0.05$; ** $P < 0.01$.

N⁶-PIA in control membranes was slightly enhanced ($21 \pm 2\%$ inhibition) but, in contrast with the data reported above, remained unchanged after a preincubation with ethanol (0.1 M) (not shown).

4. DISCUSSION

The present data confirm earlier reports [5–7,11–13] showing that ethanol at higher concentrations (>0.1 M) increases basal adenylate cyclase activity in brain cortical membranes. Rabin and Molinoff [5,6] and Tabakoff and co-workers [7,11] have ascribed this stimulatory effect to a direct action of ethanol on the catalytic site of adenylate cyclase, the activation rate of N_s and the interaction of N_s with the catalytic site. These authors, however, did not consider a possible interaction of ethanol with the N_i-mediated pathway which triggers adenylate cyclase inhibition.

Our results suggest that ethanol alters either directly or indirectly the function of this inhibitory pathway: in fact, we found that ethanol (0.1 M) was able to abolish the adenylate cyclase inhibitory response to the adenosine R_i analog N⁶-PIA, without causing any change in the characteristics of N⁶-PIA binding to the R_i-adenosine receptors. Giving added weight to this suggestion was the finding that ethanol almost completely abolished the inhibitory phase of the GTP-mediated adenylate cyclase modulation without altering the stimulatory phase (fig.2).

Two different mechanisms, at least, could explain these observations. Ethanol may act directly on the inhibitory system by decreasing the affinity of N_i for GTP and consequently by reducing the dissociation rate of N_i. Ethanol may also decrease the reassociation rate of N_s either by increasing the affinity of N_s for GTP or by reducing the affinity of α_s for the $\beta\gamma$ -subunit, leading to both a better stabilization of the α_s -catalytic subunit complex and the disappearance of adenylate cyclase inhibitory responses.

Here, abolition of the cyclase inhibitory response to N⁶-PIA by ethanol was clearly observed under experimental conditions that promote the ability of N_s to dissociate (low GTP and high Mg²⁺ concentrations) [10]. However, when these experiments were repeated under conditions favouring N_i dissociation (20 μ M GTP, 2 mM

Mg²⁺), no significant effect of ethanol on the N⁶-PIA inhibitory potency could be observed. The latter finding is consistent with recent data obtained by Rabin [12] who, also using high GTP and low Mg²⁺ concentrations, failed to observe any effect of ethanol on the adenylate cyclase inhibitory response to acetylcholine, Leu-enkephalin and morphine. These different observations therefore lead to the suggestion that besides its effects on the stimulatory pathway [5–7,11–13], ethanol is also able to interact with the N_i system, provided that the experiments are carried out under conditions favouring the N_s-mediated activatory pathway.

Whether these conditions occur in the brain in vivo remains unknown. It is thus difficult to evaluate the significance of the present findings with respect to alcoholic intoxication. It must be noted, however, that the ethanol concentration (0.1 M) inducing complete suppression of the adenosine inhibitory effect is compatible with the blood ethanol levels found after severe acute alcoholic intoxication and, in any case, much lower than those eliciting adenylate cyclase activation [5–7]. As recent studies [14] have suggested that altered adenosine functions may be implicated in some of the effects of ethanol on the central nervous system, we cannot exclude that the present findings may be relevant to some neurological manifestations of alcoholic intoxication.

ACKNOWLEDGEMENT

This work was supported by a grant (no.1984-75) from the Haut Comité d'Etude et d'Information sur l'Alcoholisme.

REFERENCES

- [1] Northup, J.K., Sternweis, P.C. and Gilman, A.G. (1983) *J. Biol. Chem.* 258, 11361–11368.
- [2] Katada, T., Northup, J.K., Bokoch, G.M., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3578–3585.
- [3] Katada, T., Bokoch, G.M., Northup, J.K., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3568–3577.
- [4] Bokoch, G.M., Katada, T., Northup, J.K., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3560–3567.
- [5] Rabin, R.A. and Molinoff, P.B. (1981) *J. Pharmacol. Exp. Ther.* 216, 129–134.

- [6] Rabin, R.A. and Molinoff, P.B. (1983) *J. Pharmacol. Exp. Ther.* 227, 551–556.
- [7] Luthin, G.R. and Tabakoff, B. (1983) *J. Pharmacol. Exp. Ther.* 228, 579–587.
- [8] Giudicelli, Y., Pecquery, R., Proven, D., Agli, B. and Nordmann, R. (1977) *Biochim. Biophys. Acta* 486, 385–398.
- [9] Cooper, D.M.F. (1982) *FEBS Lett.* 138, 157–163.
- [10] Jakobs, K.H., Aktories, K. and Schultz, G. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* 17, 135–144.
- [11] Saito, T., Lee, J.M. and Tabakoff, B. (1985) *J. Neurochem.* 44, 1037–1044.
- [12] Rabin, R.A. (1985) *Biochem. Pharmacol.* 34, 4329–4331.
- [13] Saito, T., Luthin, G.R., Lee, J.M., Hoffman, P.L. and Tabakoff, B. (1987) *Jap. J. Pharmacol.* 43, 133–141.
- [14] Dar, M.S., Mustafa, S.J. and Wooley, W.R. (1983) *Life Sci.* 33, 1363–1374.