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Regulation of the Stress-Like Protein Adenotin in PC 12 Cells by Ethanol Exposure

*Richard A. Rabin**

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY, SUNY AT BUFFALO, BUFFALO, NY 14214-3000, U.S.A.

ABSTRACT. The effects of chronic ethanol exposure on the stress-like protein adenotin were investigated using the radioligand [^{3}H]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]NECA). A 4-day exposure to 150 mM ethanol increased both the K_D and the density of [^{3}H]NECA binding sites. These changes were not due to residual ethanol as the acute addition of ethanol did not alter [^{3}H]NECA binding. Chronic ethanol exposure of A126-1B2-1 cells, which are a mutant PC 12 cell line deficient in protein kinase A (PKA), increased the cellular density of adenotin, but did not affect the K_D for the radioligand. Conversely, when PC 12 cells were exposed to 10 μM forskolin for either 2 or 4 days, the cellular density of adenotin was not altered, but the affinity of adenotin for [^{3}H]NECA was reduced significantly. An increase in K_D was not observed after a 1-hr exposure of PC 12 cells to forskolin, indicating that the reduction in affinity for the radioligand was not due simply to a PKA-mediated phosphorylation of adenotin. The present study demonstrated that chronic ethanol regulates adenotin through two different mechanisms. The ethanol-induced increase in the density of adenotin does not involve PKA, while the reduction in affinity appears to involve a cAMP-dependent mechanism. BIOCHEM PHARMACOL 51;2:183–186, 1996.

KEY WORDS. adenotin; PC 12 cell; ethanol; adenosine binding protein; protein kinase A; stress proteins

Adenotin is an adenosine binding protein that contains a high degree of homology with several stress-induced proteins including the 94-kDa glucose-regulated protein [1]. Adenotin is a homodimer composed of subunits with a molecular mass of 98 kDa, as determined by SDS–PAGE. [2]. Similar to other stress proteins, adenotin is widely distributed, being located in a variety of tissues including brain [3], platelets [4, 5], placenta [1, 2, 6], aortic smooth muscle [7], lung [8], and various cell lines [9–11]. In addition to its ubiquitous distribution, adenotin is expressed at a high cellular density. For example, in human placental membranes approximately 1% of the total protein is adenotin [2].

Exposure to ethanol induces a stress response in mammalian cells that has been suggested to be involved in the neuronal adaptation to alcohol [12–17]. Chronic exposure of NG108-15 neuroblastoma-glioma hybrid cells to ethanol increases expression of the constitutive 70-, 90-, and 110-kDa stress proteins [13, 14]. Similarly, levels of the 70-kDa stress proteins are elevated in hepatocytes from patients with alcoholic liver disease [15]. In addition to increasing the cellular levels of heat shock proteins, chronic ethanol exposure also induces members of the glucose-responsive subgroup of stress proteins, i.e. the 78- and 94-kDa glucose-regulated proteins [16, 17].

During the course of studying the cellular adaptive responses to alcohol, it was found that chronic ethanol exposure altered the density and binding properties of the stress-like protein adenotin. Furthermore, these changes appear to involve, at least in part, a cAMP-dependent mechanism.

MATERIALS AND METHODS

Materials

[^{3}H]NECA† was purchased from Amersham Life Sciences Inc. (Arlington Heights, IL). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). Heat-inactivated horse serum was obtained from JRH Bioscience (Lenexa, KS). Adenosine receptor ligands were purchased from Research Biochemical International (Natick, MA). All other chemicals were purchased from common commercial suppliers.

PC 12 and A126-1B2-1 cells were maintained at 37° under an atmosphere of 95% air–5% CO_2 in 85% Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% heat-inactivated horse serum. Chronic ethanol exposure was carried out by incubating the cells at 37° in plastic desiccators containing a 95% air–5% CO_2 atmosphere that was saturated with the appropriate concentration of ethanol. Medium was replaced after 2 days, and cells were harvested after a total of 4 days of exposure. This procedure results in a 15% loss of ethanol over a 2-day period [18]. When cells were chronically exposed to forskolin, medium was changed daily.

*Corresponding author: Richard A. Rabin, Ph.D., Department of Pharmacology and Toxicology, SUNY at Buffalo, 102 Farber Hall, Buffalo, NY 14214-3000. Tel. (716) 829-3286; FAX (716) 829-2801.

†Abbreviations: NECA, 5′-*N*-ethylcarboxamidoadenosine; MECA, 5′-*N*-methylcarboxamidoadenosine; CPCA, 5′-(*N*-cyclopropyl) carboxamidoadenosine; DPSPX, 1,3-dipropyl-8-*p*-sulfophenylxanthine; DPMA, N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine; K_D, equilibrium dissociation constant; B_{max}, binding site density; and PKA, protein kinase A (cAMP-dependent protein kinase).

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For the binding of [^{3}H]NECA to intact cells, cells were removed by trituration and washed using PBS. The binding reaction was carried out at 10° in a final volume of 300 μL PBS containing [^{3}H]NECA (25 Ci/mmol; 0.5 to 140 nM for equilibrium saturation studies; 50 nM for competition studies), 2 U/mL adenosine deaminase, approximately 2 million cells, and appropriate drugs. The incubations were terminated after 60 min using a Brandell cell harvester, and the filters were washed twice in cold 50 mM Tris buffer (pH 7.4). The amount of bound radioactivity was measured by liquid scintillation spectroscopy after incubating the filters overnight in scintillation fluid. Specific binding was defined as the difference in the amount of radioactivity bound in the absence and presence of 100 μM MECA. Data were analyzed by nonlinear regression using the program EBDA/LIGAND (Elsevier BIOSOFT).

Protein content was determined by the method of Lowry *et al.* [19].

RESULTS

The stress-like protein adenotin is characterized by: (1) a high cellular density; (2) submicromolar affinity for [^{3}H]NECA; and (3) an inability to bind N^6-substituted adenosine analogues and C^8-substituted xanthine derivatives, which is unlike the binding properties of the adenosine A_1 and A_2 receptor subtypes [4, 5, 7, 8]. Also, in contrast to the G protein-coupled adenosine receptors, stimulation of adenotin does not alter cAMP content [9]. At 10°, binding of 50 nM [^{3}H]NECA to intact PC 12 cells reached equilibrium within 45 min and was stable for at least an additional 45 min. The equilibrium binding data at the lower concentrations of [^{3}H]NECA (0.5 to 140 nM) were best described by a two-site model involving a high affinity/low density and a low affinity/high density site (Fig. 1). PC 12 cells have been shown to contain A_2, but not A_1, adenosine receptors [20, 21]. The density of high affinity [^{3}H]NECA sites in the PC 12 cells is in excellent agreement with the previously reported density of adenosine A_2 sites in these cells [22]. In addition to NECA, MECA and CPCA were capable of completely displacing specifically bound [^{3}H]NECA (Fig. 2). However, the IC_{50} values for displacement of the radioligand by DPMA and DSPX were greater than 100 μM. Similarly, the N^6-substituted adenosine analogues, N^6-cyclopentyladenosine and $R(-)N^6$-(2-phenylisopropyl)adenosine, as well as the C^8-substituted xanthine, 8-cyclopentyltheophylline had IC_{50} values much greater than 100 μM (data not shown). This pharmacological profile is consistent with [^{3}H]NECA labeling the stress-like protein adenotin.

The effects of a 4-day exposure of PC 12 cells to ethanol are shown in Fig. 3. Because the A_2 receptor represents only 6% of the total [^{3}H]NECA binding sites, the use of high concentrations of NECA (75 nM to 6 μM) to generate equilibrium saturation plots resulted in linear Scatchard plots with Hill coefficients of 1.017 ± 0.020 and 1.060 ± 0.017 for control and ethanol-treated cells, respectively. In addition, nonlinear regression analysis of these data indicated that a one-site model resulted in the best fit. Chronic ethanol exposure resulted in a statistically significant 59% increase in K_D as well as an 85% increase in B_{max} (Fig. 3). These changes were not due to the presence of residual ethanol from the chronic treatment as the acute inclusion of 150 mM ethanol did not alter either the K_D for the radioligand or the density of binding sites (Table 1).

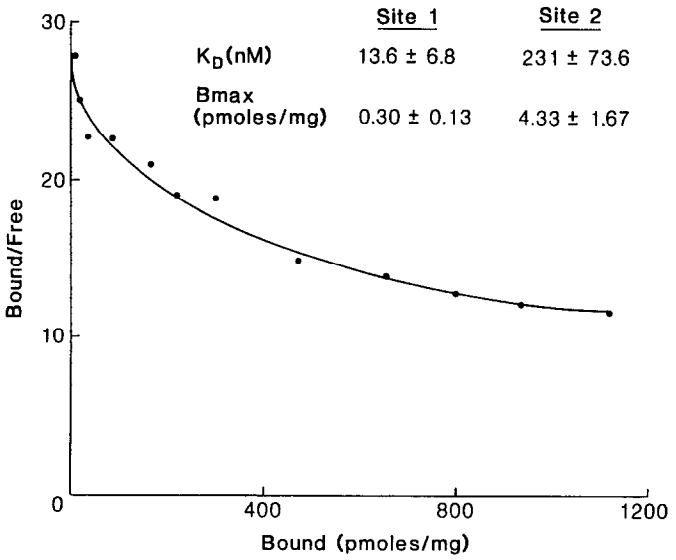

FIG. 1. Scatchard plot of the binding of [^{3}H]NECA to intact PC 12 cells. Equilibrium saturation data were generated by incubating intact PC 12 cells with various concentrations of [^{3}H]NECA (12 concentrations: 0.5 to 140 nM), and the data were plotted by the method of Scatchard. Data presented are representative of 5 separate experiments. *Inset:* K_D and B_{max} values for the two sites as determined by nonlinear regression analysis. Data are expressed as means ± SEM of the 5 separate experiments.

At least some of the adaptive cellular responses to chronic ethanol exposure appear to involve PKA [23, 24]. The role of PKA in the effects of ethanol on adenotin was investigated using A126-1B2-1 cells. These cells are a subclone of the A126-1B2 cells that were selected by resistance to dibutyryl cAMP after nitroguanidine mutagenesis of PC 12 cells [25] and have been shown to be functionally deficient in PKA activity [24, 26]. A 4-day exposure of A126-1B2-1 cells to ethanol did not alter the K_D for [^{3}H]NECA, but significantly increased the B_{max} 44% (Table 2). Conversely, a 2-day treatment of PC 12 cells with 10 μM forskolin did not alter the cellular density of adenotin, but caused a statistically significant 40% increase in the K_D (Table 3). An identical result was obtained when PC 12 cells were treated with 10 μM forskolin for 4 days (Table 3). The requirement for PKA in the ethanol-induced increase in K_D does not appear to be due simply to a phosphorylation of adenotin as a 1-hr exposure of PC 12 cells to 10 μM forskolin did not alter the affinity of adenotin for the radioligand (Table 3).

DISCUSSION

Chronic ethanol exposure induces expression of stress proteins including the constitutive 28-, 70-, and 110-kDa heat shock proteins as well as the 78- and 94-kDa glucose-regulated pro-

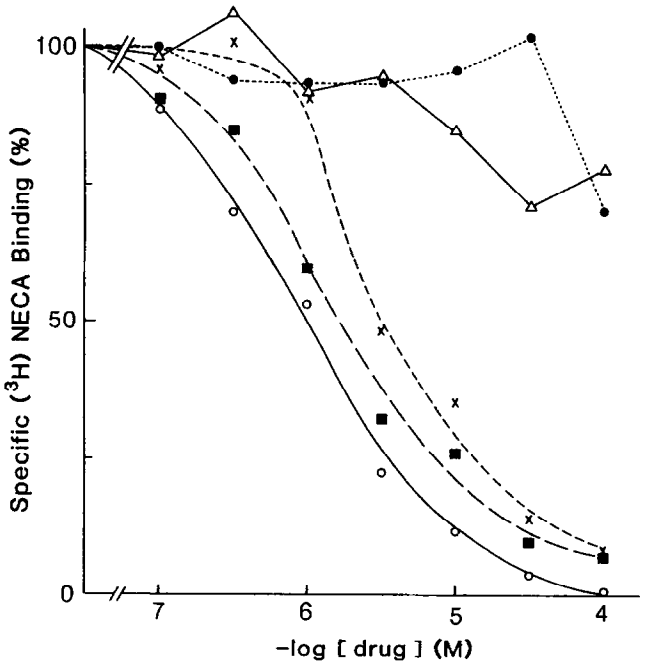

FIG. 2. Pharmacological profile of [^{3}H]NECA binding. Intact PC 12 cells were incubated with 50 nM [^{3}H]NECA and various concentrations of MECA (○), CPA (■), unlabeled NECA (X), DPSPX (●) and DPMA (△). Data are plotted as a percent of the amount of radioligand specifically bound in the absence of unlabeled competing ligand. Data are plotted as the means of 3–4 separate experiments.

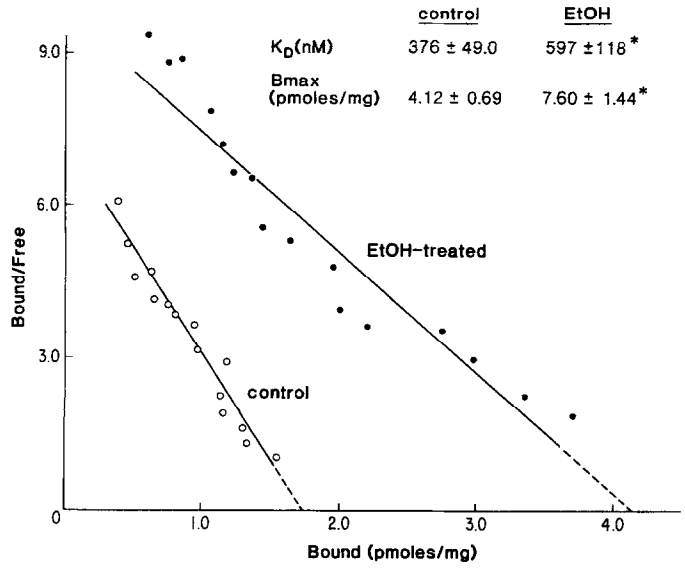

FIG. 3. [^{3}H]NECA binding: Chronic ethanol exposure of PC 12 cells. PC 12 cells were exposed to 150 mM ethanol for 4 days. Equilibrium saturation data were generated by incubating the cells with 50 nM [^{3}H]NECA plus various concentrations of unlabeled NECA (16 concentrations: 25 nM to 5.95 μM). Data are plotted by the method of Scatchard, and are representative of 8 separate experiments. *Inset:* K_D and B_{max} values determined by nonlinear regression are expressed as means ± SEM of the 8 separate experiments. Key: (*) $P < 0.05$ (paired *t*-test) compared with the corresponding value in control cells.

TABLE 1. Effects of Acute Ethanol on [^{3}H]NECA Binding

	Control	Ethanol
K_D (nM)	257 ± 28	315 ± 27
B_{max} (pmol/mg)	2.09 ± 0.12	2.48 ± 0.26
Hill coefficient	1.00 ± 0.02	1.00 ± 0.02

Intact PC 12 cells were incubated for 60 min with various concentrations of [^{3}H]NECA (50 nM [^{3}H]NECA plus 14 concentrations of unlabeled NECA: 50 nM to 1.95 μM) in the absence and presence of 150 mM ethanol. The equilibrium saturation data were analyzed by nonlinear regression as described in Materials and Methods. Data are expressed as means ± SEM of 6 separate experiments.

teins [12–17]. In the present study, chronic ethanol exposure increased the number of [^{3}H]NECA binding sites, which is indicative of an increase in the amount of adenotin in the cells. However, alternative explanations such as a change in a regulatory protein cannot be ruled out at the present time. The increase in the expression of adenotin was accompanied by a reduction in the affinity of the protein for the radioligand. These changes in [^{3}H]NECA binding can be entirely attributed to ethanol-induced alterations in the properties of adenotin. First, chronic ethanol exposure does not alter the properties of the A_2 receptor in PC 12 cells as measured using [^{3}H]CGS 21680 [22]. Second, with the assay conditions used, [^{3}H]NECA binding to the A_2 receptor represents a very minor component of the total amount of radioligand bound.

Chronic ethanol exposure appears to be regulating adenotin through two different mechanisms. An increase in B_{max} was observed after chronic ethanol exposure of the PKA-deficient A126-1B2-1 cells, but not after chronic treatment with forskolin. This indicates that activation of PKA alone does not mediate the ethanol-induced increase in the amount of adenotin. Conversely, the ethanol-induced increase in the K_D for the radioligand appears to involve a cAMP-dependent mechanism. The K_D for [^{3}H]NECA was not altered after chronic ethanol exposure of A126-1B2-1 cells, but chronic treatment of wild-type PC 12 cells with forskolin did reduce the affinity of adenotin for the radioligand. The role of cAMP in the ethanol-induced increase in K_D is not known. Phosphorylation of heat shock protein 27 alters the ability of this stress protein to regulate microfilament dynamics [27]. However, because a 1-hr exposure to forskolin did not alter the binding

TABLE 2. Effects of Chronic Ethanol Treatment on the Binding Properties of [^{3}H]NECA in the PKA-Deficient A126-1B2-1 Cells

	Control	Ethanol
K_D (nM)	388 ± 36	341 ± 35
B_{max} (pmol/mg)	1.83 ± 0.19	2.64 ± 0.38*
Hill coefficient	1.01 ± 0.01	1.02 ± 0.03

After a 4-day exposure to 150 mM ethanol, intact A126-1B2-1 cells were incubated with 50 nM [^{3}H]NECA and various concentrations of unlabeled NECA (14 concentrations: 50 nM to 1.95 μM). The equilibrium saturation data were analyzed by nonlinear regression as described in Materials and Methods. Data are expressed as means ± SEM of 6 separate experiments.

* $P < 0.05$ (paired *t*-test) compared with control.

TABLE 3. Effects of *in vivo* exposure to forskolin on [^{3}H]N-ECA binding

	Control	Forskolin
K_D (nM)		
1 hr	218 ± 35	215 ± 18
2 days	146 ± 14	205 ± 17*
4 days	177 ± 16	247 ± 25*
B_{max} (pmol/mg)		
1 hr	2.06 ± 0.25	2.07 ± 0.18
2 day	3.03 ± 0.35	3.34 ± 0.32
4 day	1.73 ± 0.13	2.03 ± 0.12

PC 12 cells were incubated in complete medium containing 10 μM forskolin for 1 hr or maintained in this forskolin-supplemented medium for either 2 or 4 days. Equilibrium saturation data were generated by incubating cells with 50 nM [^{3}H]NECA and various concentrations of unlabeled NECA (10 concentrations: 0 to 1.5 μM). Data are expressed as means ± SEM of 3 (1-hr exposure), 6 (2-day exposure) or 9 (4-day exposure) separate experiments.

* $P < 0.05$ (Student's *t*-test) compared with the appropriate control.

of adenotin, the reduced affinity of this protein does not appear to involve a PKA-mediated phosphorylation of adenotin itself.

Stress proteins are involved in a variety of normal cellular functions as well as playing a key part in the adaptive response to environmental insults such as ethanol exposure. The results of the present study indicate that the ubiquitous and abundant stress protein adenotin can be regulated and should be included as an ethanol-responsive gene. Although cAMP does not appear to be involved in the expression of adenotin, the present results suggest a PKA-dependent phosphorylation may play a role in the function of adenotin.

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