BASAL AND ISOPROTERENOL-STIMULATED CYCLIC-ADENOSINE MONOPHOSPHATE LEVELS IN MOUSE HIPPOCAMPUS AND LYMPHOCYTES DURING ALCOHOL TOLERANCE AND WITHDRAWAL

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Abstract — Aims: Basal and isoproterenol-stimulated levels of cyclic-adenosine monophosphate (cAMP) were investigated in the brain (hippocampus) and in the lymphocytes of mice rendered tolerant to and, physically dependent on, ethanol. Methods: cAMP was measured with radioimmunoassay. Tolerance to, and physical dependence on, ethanol were induced by a 14-day ingestion of ethanol in drinking water. Upon replacing ethanol with water, ethanol withdrawal was precipitated and measured by the intensity of withdrawal-induced hyperexcitability and seizures. Results: Basal (non-stimulated) levels of cAMP — both in the hippocampus and in the lymphocytes — were significantly reduced in the alcohol-drinking tolerant and physically dependent animals, but significantly increased 24 h after the onset of withdrawal. Isoproterenol resulted in a dose-dependent stimulation of cAMP in all groups investigated (control, tolerant/physically dependent, withdrawal), however, the magnitude of isoproterenol-induced net increase was significantly lower in the tolerant, and higher in the ethanol-withdrawn, animals. Conclusions: The major finding of the present experiments is that there was a significant positive correlation between basal cAMP levels in brain and lymphocytes versus the intensity of withdrawal hyperexcitability in ethanol-addicted mice.

INTRODUCTION

Widespread evidence indicates that the sympathetic nervous system innervates all lymphoid organs and that the catecholamines (noradrenaline and adrenaline) modulate several immune parameters (for review, see Elenkov et al., 2000). Catecholamines mediate their effects on target cells via stimulation of α- and β-adrenergic receptors. β-Adrenoreceptors are integrated into plasma membranes in various tissues throughout the human and animal body, and virtually all lymphoid cells express β-adrenoreceptors (Khan et al., 1986). Catecholamines directly transduce their signal into a cell by activating G-protein-coupled receptors that modulate adenyl cyclase (AC). AC, on the other hand, changes intracellular cyclic-adenosine monophosphate (cAMP) levels, which subsequently alters cAMP-dependent protein kinase A (PKA). Despite some continuous debate, most investigators agree that β-adrenoreceptor function on lymphocytes is representative of β₂-adrenoreceptor function in other organs, especially the brain and the heart (Brodde et al., 1987).

There is increasing evidence that the cAMP signalling system and PKA are involved in mediating some effects of acute and chronic cellular responses to ethanol (Coe et al., 1996a; Diamond and Gordon, 1997; Szegedi et al., 1998; Dahmen et al., 2000). Accordingly, ethanol given acutely increases adenosine receptor-stimulated CAMP levels (Pandey et al., 2001). AC activity is also increased in the presence of ethanol (Rabani and Tabakoff, 2001). The magnitude of ethanol’s action on AC depends on the isoform of AC expressed in a particular cell type. Type VII AC demonstrates the greatest potency of activation in the presence of ethanol. Recent results of Yoshimura and Tabakoff (1999) indicate that, in the presence of this particular Type VII isoform of AC, moderately intoxicating concentrations of ethanol will significantly potentiate the transmitter-mediated activation of the cAMP signalling cascade.

Examples from both human and animal research indicate that high levels of ethanol drinking are often associated with resistance to the physiological effects of this drug (Schuckit, 1994; Kurtz et al., 1996). Several earlier studies have demonstrated that chronic ethanol treatment decreases various components (neurotransmitter receptors, G proteins, AC, PKA) of the cAMP signal transduction cascade, especially in the brain (Hoffman and Tabakoff, 1990), but also in lymphocytes (Diamond et al., 1987). Activation of the cAMP signalling cascade — especially in the brain — may have important implications for the mechanisms of neuroadaptation leading to tolerance and, physical dependence on, ethanol.

Cessation of chronic ethanol consumption is often accompanied by signs and symptoms characteristic of ethanol withdrawal syndromes (Koob and Bloom, 1988; Harris and Buck, 1990). Alterations in the various steps of the cAMP signal transduction pathway in the brain and in other cell systems, including blood lymphocytes, during ethanol tolerance and dependence, have been demonstrated by several investigators (Diamond et al., 1987; Pandey et al., 1999).

Although changes in the basal and stimulated CAMP levels have been repeatedly reported in human alcoholics and also in ethanol-dependent experimental animals, some of these effects, especially those in humans, might be age-related (Dahmen et al., 2000), or determined by individual genetic differences (Gordon et al., 1990; Diamond et al., 1991). More recent results by Szegedi et al. (1998), however, indicate state-dependent changes in the activity of AC during alcohol withdrawal in male patients.

The brain and the immune system — the two ‘supersystems’ (Tada, 1997) — are the major adaptive systems of the body, which are involved in functionally relevant cross talk, whose main function is to maintain homeostasis. Whereas cellular dysfunctions of the immune system can be directly measured...
in human alcoholics, those of the brain cannot be studied at the cellular level in humans. The present experiments were undertaken to investigate state-dependent changes in basal and isoproterenol-stimulated cAMP levels in the brain as well as in peripheral lymphocytes of mice rendered tolerant to, and physically dependent on, ethanol. An attempt has been made to correlate these biochemical changes with the severity of ethanol withdrawal-induced hyperexcitability.

MATERIALS AND METHODS

Experimental animals and the induction of tolerance to and physical dependence on ethanol and the measurement of withdrawal hyperexcitability

Male inbred CFLP mice (Gödöllő, Hungary), weighing 35 ± 5 g (mean ± SD), were used in the experiments (for details, see Kovács, 1993, 2000). Animals were kept ad libitum on standard chow (Charles River, Hungary) in groups of 15. The various treatment groups are described in Table 1. Animals rendered tolerant to, and physically dependent on, ethanol, received ethanol in the drinking water [5% (v/v) for 1 week, then 7% for the second week]. On day 15, tolerant and physically dependent mice also received 7% ethanol for drinking, and thus withdrawal was not precipitated. In another group of tolerant and physically dependent mice, ethanol withdrawal was tested. In this group, ethanol was replaced by isocaloric sucrose on day 15. Five hours later, withdrawal hyperexcitability (handling-induced convulsion) was scored according to Goldstein (1972): 0, no convulsion; 1, facial grimace (after 180 degree spin); 2, tonic convulsion (after 180 degree spin); 3, tonic-clonic convulsion (after 180 degree spin); 4, tonic convulsion (when lifted by tail); 5, tonic-clonic convulsion (when lifted by tail); 6, severe tonic-clonic convulsions of long duration (when lifted by tail); 7, severe tonic-clonic convulsions of long duration (before lifted by tail); 8, severe tonic-clonic convulsions ending with death (apparently no animal died during the withdrawal procedure). Ethanol-withdrawn animals were killed 24 h after the beginning of the withdrawal procedure.

Control animals received an isocaloric sucrose solution for drinking throughout the whole treatment period. At the end of the experiment, mice were decapitated. The brain was removed and the trunk blood was collected for biochemical studies (see below).

Blood-ethanol concentration

Blood-ethanol levels were measured with the UV photometric method of Boehringer Diagnostica Mannheim GmbH. The assay principle is based on measurement of the increase in NADH on enzymatic dehydrogenation of NAD⁺. Samples were measured on an automated clinical chemistry analyser (Hitachi-911) at a wavelength of Hg 365 nm. Results are expressed as mg ethanol/ml blood.

Measurement of isoproterenol-stimulated cAMP production

Hippocampus. Upon decapitation, the brain was quickly removed and the dorsal hippocampus was dissected on ice with a forceps from 1-mm-thick coronal sections of the brains. Principally, the landmarks identified by Franklin and Paxinos (1997) were used. The dorsal hippocampi of six animals were pooled for the cAMP analysis. The hippocampal tissue was cut into slices with a tissue chopper and placed in ice-cold artificial CSF (Watabe et al., 2000). Tissue suspensions were divided into aliquots. Graded amounts (0, 10 and 100 nM) of isoproterenol (isoproterenol hemisulphate; Sigma) dissolved in distilled water containing theophylline (10 mM) and ascorbic acid (1 mg/ml), dissolved in a volume of 10 μl, were added to the tissue suspension aliquots and incubated at 37°C for 15 min. Isoproterenol, in a range of 1–1000 nM, resulted in a linear stimulation curve of cAMP formation.

Following incubation, the standard assay methodology of the commercial Biotrak cAMP(125I) kit (Amersham Life Sciences) was strictly followed. Brain tissue was homogenized in ice-cold trichloroacetic acid [6% (w/v)] to give a 10% (v/v) homogenate. An aliquot of 100 μl was taken for the measurement of proteins (Lowry et al., 1951). Samples were centrifuged at 4°C (2000 g, 15 min). The supernatant was washed four times with 5 vol of water-saturated diethyl ether. The upper ether layer was discarded after each wash. The aqueous extract was evaporated in a nitrogen flow at 40°C.

Lymphocytes. For the separation of lymphocytes, the method of Diamond et al. (1987) was followed. Trunk blood of six mice (the same six as collected for one hippocampal sample) was pooled and collected into heparinized vials (286 USP heparin/vial) in order to obtain ca. 10 ml of blood for one sample. The blood sample was gently layered onto 5 ml of Ficoll solution (density = 1.077 g/cm³). After centrifugation (at 4°C, 400 g, for 30 min), the mononuclear cells, including B and T lymphocytes and monocytes, were removed from the

<table>
<thead>
<tr>
<th>Pretreatment for 2 weeks</th>
<th>Drinking on day 15</th>
<th>Withdrawal hyperexcitability tested</th>
<th>Measured biochemical parameter after decapitation</th>
<th>Group name (group names as they appear on the figures and tables)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocaloric sucrose in water</td>
<td>Water not removed</td>
<td>No</td>
<td>cAMP, blood ethanol concentration</td>
<td>Control (Control)</td>
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<tr>
<td>Ethanol in drinking water (for details, see Materials and methods)</td>
<td>Ethanol not replaced with water</td>
<td>No</td>
<td>cAMP, blood ethanol concentration</td>
<td>Tolerant/physically dependent (Tolerant)</td>
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<tr>
<td>Ethanol in drinking water (for details, see Materials and methods)</td>
<td>Ethanol replaced with water for 24 h</td>
<td>Yes, 5 h after removal of ethanol</td>
<td>cAMP, blood ethanol concentration</td>
<td>Withdrawal (Withdrawal)</td>
</tr>
</tbody>
</table>

*All animals were decapitated between 11.00 and 13.00.
HIPPOCAMPAL AND LYMPHOCYTE CAMP AND ALCOHOL ADDICTION

RESULTS

The 14-day-long ethanol treatment resulted in the development of tolerance to, and physical dependence on, alcohol, as indicated by the appearance of medium severe ethanol withdrawal symptoms on day 15 (mean withdrawal hyperexcitability score 3.1 ± 0.1). The withdrawal scores of the 60 mice tested for withdrawal ranged between 2 and 5 on a scale of 0–8 (Fig. 1).

The blood-ethanol concentration in animals rendered tolerant to, and physically dependent on, ethanol was significantly elevated (\( F = 386.3; \text{df} = 2; P < 0.001 \)) 14 days after the beginning of a continuous ethanol liquid diet. Twenty-four hours after replacement of ethanol with water, i.e. after the onset of withdrawal symptoms, ethanol completely disappeared from the blood (Table 2).

Univariate ANOVA analysis of the basal and isoproterenol-stimulated cAMP levels (Fig. 2) indicated significant differences among various treatment groups for the hippocampus (\( F = 128.96; \text{df} = 2; P < 0.001 \)) as well as for lymphocytes (\( F = 11.96; \text{df} = 8; P < 0.001 \)). Both in the hippocampus (\( F = 128.96; \text{df} = 2; P < 0.001 \)) and in lymphocytes (\( F = 28.74; \text{df} = 2; P < 0.001 \)), there was a significant effect on cAMP of ethanol tolerance/physical dependence as well as of ethanol withdrawal. Isoproterenol stimulation also induced significant effects in the hippocampal tissue (\( F = 111.27; \text{df} = 2; P < 0.001 \)) as well as in the lymphocytes (\( F = 16.57; \text{df} = 2; P < 0.001 \)).

Both in the hippocampus (\( F = 47.10; \text{df} = 2; P < 0.001 \)) as well as in the lymphocytes (\( F = 10.78; \text{df} = 2; P < 0.001 \)) of mice rendered tolerant to, and physically dependent on, ethanol, one-way ANOVA analysis revealed significant differences in the basal (non-stimulated) cAMP levels. In both tissues, basal cAMP levels were significantly lower in the ethanol-tolerant animals (post hoc analysis: \( P < 0.05 \) for both tissues), but significantly higher in the withdrawal group (post hoc analysis: \( P < 0.05 \) for both tissues), than in the control animals. The isoproterenol-stimulated cAMP levels significantly elevated (\( F = 386.3; \text{df} = 2; P < 0.001 \)) 14 days after the beginning of a continuous ethanol liquid diet. Twenty-four hours after replacement of ethanol with water, i.e. after the onset of withdrawal symptoms, ethanol completely disappeared from the blood (Table 2).

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were also statistically lower (P < 0.05) in the tolerant, and higher in the withdrawal groups, if compared to the respective stimulated levels in the control group (Fig. 2).

The net increase of cAMP production in response to stimulation with 10 nM isoproterenol was also statistically different among the three treatment groups in both the hippocampus (F = 35.26; df = 2; P < 0.001) as well as in the lymphocytes (F = 3.73; df = 2; P < 0.05). Tolerant and physically dependent animals were characterized by a lower (P < 0.05), whereas the withdrawn animals by a higher (P < 0.05) net cAMP increase (Fig. 3). Similar results were found, when the stimulatory effect of 100 nM isoproterenol was calculated (data not shown).

In order to perform regression analysis between withdrawal hyperexcitability and the cAMP formation, both hippocampal tissues and the trunk blood of ethanol-withdrawn animals were pooled from six experimental mice exhibiting the same intensity of withdrawal hyperexcitability. Linear regression analysis revealed a significant positive correlation between basal (non-stimulated) cAMP levels in mouse hippocampal tissue (F = 99.98; P < 0.001) and lymphocytes (F = 61.54; P < 0.001) versus the intensity of withdrawal hyperexcitability (Fig. 4).

**DISCUSSION**

Adenosine receptors are membrane-bound proteins which appear to mediate some effects of ethanol in the brain and thus the cAMP signalling pathway is a major target for ethanol in the intact cell (Diamond and Gordon, 1997). Alcoholism
HIPPOCAMPAL AND LYMPHOCYTE cAMP AND ALCOHOL ADDICTION

Fig. 4. Linear regression analysis of basal (non-stimulated) cAMP levels in the hippocampus and in lymphocytes with withdrawal hyperexcitability in mice rendered physically dependent on ethanol.

Individual points represent the data of the hippocampi and lymphocytes pooled from six ethanol-dependent mice, killed 24 h after the onset of ethanol withdrawal. The horizontal axis indicates the mean basal (non-stimulated) cAMP levels, the vertical axis depicts the intensity of withdrawal hyperexcitability. The horizontal line shows the mean value of ethanol withdrawal hyperexcitability (x = 3.1). The linear regression line and the reference intervals of the regression line are also indicated on the figure.

causes serious neurological diseases that may be due, at least in part, to the ability of ethanol to interact with neural cell membranes and change neuronal functions. It has been hypothesized (Mochly-Rosen et al., 1988) that cellular adaptation to ethanol involves a reduction of neuronal cAMP production, which in turn may partially account for the appearance of tolerance and physical dependence. Lymphocytes also have adenosine receptors, and their activation causes increases in cAMP levels.

A 14-day-long continuous ingestion of ethanol (in drinking water) results in the appearance of tolerance to, and physical dependence on, ethanol in the inbred CFLP mice, investigated in the present experiments. In the alcohol-dependent organism, disturbances of the autonomic nervous system are well known. Accordingly, signs of overactivity of the sympathetic nervous system characterize the alcohol-withdrawal syndrome. An increased release of catecholamines (adrenaline and noradrenaline) is associated with certain symptoms of withdrawal, such as hyperexcitability, tremulousness, paroxysmal sweats, increased blood pressure and increased heart rate (Linnoila et al., 1987; Kovács et al., 2002). Abnormalities in central nervous catecholaminergic systems in alcoholism have been described, and significant attempts have been made to correlate results with various characteristics of alcoholism, such as severity, duration of abstinence, etc. Rapid and severe changes in glucose metabolism, plasma potassium level (Laso et al., 1990), liver oxygen extraction (Hadengue et al., 1994), or those in various cellular and humoral immune parameters (Hasko et al., 1998; Ramer-Quinn et al., 2000) might be directly related to, and influenced by, peripheral levels of catecholamines. The increased incidence of sudden death repeatedly reported in chronic alcoholism may also be partly related to the sympathetic nervous system and to peripheral catecholamines which result in an increased electrical vulnerability of the heart (Patel et al., 1991; Maki et al., 1998). Altered neurotransmission and adrenal release of catecholamines may subsequently change adrenergic receptor functions (for review, see Fahlke et al., 1999).

It has been found (MacGregor et al., 1996) that cAMP production in lymphocytes depends on the stimulation of β2-adrenergic receptors and that isoproterenol is one of the most efficacious agonists at these receptors. Once activated, the β-receptor complex generates cAMP from its precursor, adenosine triphosphate. cAMP then acts as an intracellular mediator for many enzymatic reactions that ultimately increase intracellular calcium and also stimulates the expression of numerous genes via the PKA-mediated phosphorylation of the cAMP response element binding protein (Pandey et al., 2001).

There is a general consensus in the literature that the AC signal transduction pathway is a target of acute and chronic ethanol actions (Pauly et al., 1999). The present results confirm previous findings of other investigators (Diamond et al., 1987; Pandey et al., 1999) indicating that cAMP levels of peripheral lymphocytes are affected by physical dependence to ethanol and also by the appearance of ethanol-withdrawal symptoms. Accordingly, reduced basal and isoproterenol-stimulated cAMP levels were measured in the alcohol tolerant and physically dependent animals during a period of regular ethanol intake, when ethanol withdrawal symptoms did not appear. The detailed mechanism of decreased cAMP production is unknown, although it is generally considered to be part of the organism’s adaptation to repeated ethanol challenge. Of interest is the observation that the levels of circulating catecholamines are also affected during ethanol tolerance (Linnoila et al., 1987; Kovács et al., 2002), suggesting that changes in the β-adrenoreceptor density might be one of the underlying reasons. However, this may not be the only responsible mechanism, since some lymphocyte subsets, such as the B cells, may have a high prevalence of low affinity receptors, which are weakly coupled to AC (Elenkov et al., 2000). The relative ratio of various lymphocyte subsets and Th1/Th2 functions may also change during the development of ethanol tolerance (Starckenburg et al., 2001). Decreased cAMP production may also be explained by a decrease in the amount and/or activity of several key proteins in this pathway (Mochly-Rosen et al., 1988; Williams and Kelly, 1993; Dohrman et al., 1996). An
adaptive decrease in receptor-stimulated cAMP levels, similar to that in lymphocytes, also occurs in neuronal cells, e.g. in cultured neuroblastoma × glioma cells (Coe et al., 1996b). Taken together, there appears to be a correlation between ethanol-induced heterologous desensitization of cAMP production in hippocampal cells and peripheral lymphocytes and the development of tolerance to, and physical dependence on, ethanol in alcohol-addicted mice.

In the experimental model described above, medium to severe symptoms of withdrawal hyperexcitability develop upon ethanol withdrawal. Withdrawal symptoms fully develop within 4–5 h after ethanol withdrawal and remain constant for 24 h (Kovács, 1993, 2000; Kovács et al., 2002). During the development of ethanol withdrawal in the physically dependent animals, both basal and isoproterenol-stimulated cAMP levels increased to a substantial extent. Similar findings were reported in young, but not in old, human alcoholic patients after alcohol detoxification (Dahmen et al., 2000). In human patients, however, changes in the cAMP signal transduction system may reflect not only age-related, but presumably also genetic differences in alcoholism (Gordon et al., 1990). Thus, individual differences in patients, specifically related to the severity of ethanol addiction are difficult to study. The novelty of the present paper is the finding that there is a correlation between cAMP production in hippocampal cells, or peripheral lymphocytes, versus the severity of ethanol withdrawal-induced hyperexcitability in inbred mice of the same age. The individual differences in the severity of withdrawal hyperexcitability were large enough to calculate such a correlation, although mice treated similarly with alcohol exhibited a statistically homologous appearance of withdrawal symptoms. Mice with lower basal and isoproterenol-stimulated cAMP production exhibited less severe withdrawal symptoms. These individual differences in cAMP production are likely to be related to individual differences in the rate of ethanol dependence, rather than to other biological or genetic variables.

Recently, Szegedi et al. (1998) examined AC activity in peripheral lymphocytes of male alcohol-dependent patients at various time points during the clinical course of detoxification. These authors detected a significant decrease in basal and stimulated AC activity during withdrawal, with lowered AC activities in a vast majority of patients. This effect resolved after detoxification, indicating rapid and marked state-dependent changes during the course of detoxification. Our present observations confirm the state-dependent character of the cAMP signalling system in lymphocytes; however, an increased, rather than a decreased (Szegedi et al., 1998), basal and stimulated cAMP formation has been observed during the withdrawal period in mice. Apart from the potential species-specificity, which is rather unlikely in this case, human alcoholics are usually characterized by a years-long duration of alcohol intake, and often also by repeated spontaneous withdrawal procedures. Widespread changes in various lymphocyte subsets in human chronic alcoholic patients, even in those without alcoholic liver disease, have been described as well (Cook et al., 1995; Laso et al., 1996). These patients are frequently immuno-deficient, have polyclonal hyper-gammaglobulinemia, and often have auto-antibodies (Cook et al., 1996). Several changes in immune parameters have also been described in mice, even following a short-term ethanol-containing liquid diet treatment (Gallucci et al., 1994; Hsiung et al., 1994). However, the percentage of the lymphocyte subsets (e.g. natural killer cells in the blood and in the spleen) remained relatively more constant during a short-term (2-week) alcohol exposure (Blank et al., 1993). Further experiments are needed to elucidate the exact mechanism of this discrepancy between human and animal experiments.

In conclusion, the present results confirm previous findings of other laboratories showing marked changes in the cAMP production in hippocampal cells and of peripheral lymphocytes during the development of physical dependence on ethanol and ethanol withdrawal. However, this study provides evidence that there is a close correlation between the severity of ethanol withdrawal and cAMP production. Both human and animal studies suggest that the β-adrenoreceptor-cAMP/PKA pathway is involved in various functions of the lymphocytes, including potentiation of type 2 cytokine production and inhibition of type 1 cytokine production (Elenkov et al., 2000; Riese et al., 2000). Withdrawal-related individual differences in cAMP production of lymphocytes, therefore, may have various subsequent functional consequences.

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