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European Journal of Pharmacology 383 (1999) 203–208

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Chronic ethanol enhances muscarinic receptor-mediated activator protein-1 (AP-1) DNA binding in cerebellar granule cells

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Received 15 March 1999; received in revised form 23 August 1999; accepted 27 August 1999

Abstract

In this study we investigated effects of acute and chronic ethanol exposure on carbachol-induced activator protein-1 (AP-1) DNA binding in rat cerebellar granule cells. Acute ethanol application did not alter, whereas chronic ethanol exposure potentiated the carbachol-induced AP-1 DNA binding. The protein composition of the AP-1 transcription factor complex activated by carbachol stimulation of muscarinic receptors was analysed in control and chronic ethanol-exposed cells using a supershift assay with specific antibodies against c-Fos, Fos B, c-Jun, Jun B and Jun D proteins. Supershift analysis revealed that the carbachol-induced AP-1 complex was composed predominantly of Jun D and c-Fos. The composition of the AP-1 complex activated by carbachol in chronic ethanol-exposed cells did not differ from control. These findings indicate that chronic ethanol treatment can modulate carbachol-induced AP-1 DNA binding activity in cerebellar granule cells.

Keywords: Muscarinic receptor; Carbachol; AP-1 (activator protein-1); Ethanol; Transcription factor; Cerebellar granule cell; (Rat)

1. Introduction

Accumulated knowledge suggests that activation of brain muscarinic receptors plays an important role in the modulation of neurochemical processes involved in the regulation of synaptic plasticity (Hagan and Morris, 1988; Hirotsu et al., 1989; Burgard and Sarvey, 1990). Four different muscarinic receptor subtypes, named M_{1-4}, have been functionally characterised (Dorje et al., 1991; Caulfield, 1993). All subtypes of muscarinic receptors interact with G-proteins that regulate a variety of effector proteins within the cell. For example, muscarinic M_2 and M_4 receptor stimulation produces inhibition of adenylyl cyclase activity (Caulfield et al., 1993). In contrast, stimulation of muscarinic M_1 and M_3 receptors mainly activate phospholipase C and leads to hydrolysis of polyphosphoinositides and mobilization of intracellular Ca^{2+} (Hosey, 1992; Caulfield, 1993; Caulfield et al., 1993). This effect results in a variety of Ca^{2+}-mediated events, directly or via protein phosphorylation (for recent reviews, see Haddad and Rousell, 1998; Pandey, 1998).

Many laboratories have shown that stimulation of muscarinic receptors increases the expression of immediate early genes like *fos* and *jun*. Thus, specific patterns of muscarinic receptor-mediated immediate early gene expression have been observed in a multitude of experimental models both in vivo and in vitro including primary cultures of cerebellar granule cells (Fukamuchi et al., 1993). Induction of immediate early genes is often associated with a long-lasting enhancement of activator protein-1 (AP-1) transcription factor DNA binding activity (Morgan and Curran, 1991; Pennypacker et al., 1995). The AP-1 transcription factor is a dimer composed of Fos and Jun proteins and their cognates which bind with high affinity to corresponding regulatory elements in the promoter region of many genes thereby altering their expression, an effect that is believed to play a significant role in the long-term modulation of vital cellular functions (Chiu et al., 1989; Angel and Karin, 1991). Moreover, it is suggested that AP-1-produced alterations in gene expression may play a crucial role in long-term neuroadaptive changes in the brain associated with the development of tolerance

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00632-9

and dependence to addictive drugs (Nestler et al., 1993; Hou et al., 1996).

Acutely administered ethanol alters the muscarinic receptor-mediated signal transduction by inhibiting phospholipase C (Hoffman et al., 1986; Sanna et al., 1994). Following in vivo chronic ethanol administration, an up-regulation of the number of muscarinic receptors has been observed (Tabakoff et al., 1979; Hoffman et al., 1986; Pietrzak et al., 1990).

Previously, we have shown that acute and chronic ethanol exposure, respectively, inhibited or potentiated *N*-methyl-D-aspartate (NMDA) glutamate receptor-mediated activation of AP-1 DNA binding in primary cultures of rat cerebellar granule cells (Cebers et al., 1996; Hou et al., 1996). In a recent report, we demonstrated that muscarinic agonist carbachol induces a marked concentration-dependent enhancement of AP-1 DNA binding predominantly via muscarinic M_3 receptors, and that pre-treatment with carbachol potentiates the activation of AP-1 DNA binding caused by stimulation of ionotropic glutamate receptors in cultured neurons (Cebers et al., 1999).

In the present study, we have examined the effects of acute and chronic ethanol exposure on carbachol-stimulated AP-1 DNA binding. Furthermore, we analysed the protein composition of the AP-1 transcription factor complex activated by the stimulation of muscarinic receptors in control and chronic ethanol-exposed cultures using a supershift assay with specific antibodies against c-Fos, c-Jun, Fos B, Jun B or Jun D proteins.

2. Materials and methods

2.1. Cerebellar granule cell cultures

All experiments were approved by the local Ethical Committee for animal experiments. Primary cultures of granule cells were prepared from 8-day-old Sprague–Dawley rat cerebellum as previously described (Levi et al., 1984) with some modifications (Cebers and Liljequist, 1995). Briefly, after trypsinization cells were dispersed by trituration and plated (1×10^6 cells/ml) in Nunc 60-mm dishes, or in 6- or 24-well plates coated with 5 μg/ml of poly-L-lysine. Cells were cultured for 8 days at 37°C in an atmosphere of 5% CO_2/95% air in a Basal Eagle's Medium with Earle's salts (Life Technologies, Scotland) supplemented with 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin. Cytosine-β-arabinofuranoside (10 μM) was added 24 h after plating to limit the number of non-neuronal cells to less than 5%. The culture medium was not changed until cultures were used in the experiments.

2.2. Chronic ethanol exposure

Each batch of cultured cerebellar granule cells was divided into control and ethanol-treatment group. Ethanol (10 μl of 10 M ethanol solution) was added once a day directly into the culture medium ($V = 2$ ml). Ethanol treatment was continued for 3 days (last addition 24 h prior to experiment). Control group received sterile saline according to the same protocol. Culture plates (also control) were carefully sealed with parafilm in order to prevent the evaporation of ethanol. Ethanol concentration was measured (Procedure #333-UV, Sigma) from culture medium samples taken 24 h after each consecutive addition of ethanol and found to be (in mM) 43.6 ± 0.6; 71.1 ± 1.3; 98.5 ± 2.4 after, respectively, the first, second and third addition of ethanol.

2.3. Drug treatment

In control cells, acute effects of carbachol and ethanol on AP-1 DNA binding activity were examined by adding small volumes of concentrated stock solutions directly to the cell culture media and incubating for 2 h in the CO_2-thermostat until cells were collected and lysed. In chronic ethanol-treated cells, acute experiments were started by replacing ethanol-containing medium with 1 ml medium from sister-cultures that were not exposed to ethanol, thereafter these cultures were exposed to various concentrations of acutely added carbachol as described above.

2.4. Preparation of cell extracts and electromobility shift assay (EMSA)

Cell extracts were prepared as previously described (Hou et al., 1996). Briefly, cells from one 60-mm dish were washed with 6 ml ice-cold phosphate-buffered saline (PBS), centrifuged at $3000 \times g$ for 5 min, and suspended in Dignam's buffer (20 mM HEPES, pH 7.9; 0.42 M NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 0.5 mM dithiothreitol) supplemented with 0.2% NP-40 and the following protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride; 10 μg/ml leupeptin, 0.1 mM aminobenzamidine, 10 μg/ml aprotinin, 1 μg/ml pepstatin A. Lysates were vortexed, incubated on ice for 20 min, and then centrifuged for 10 min at $20000 \times g$. The supernatants were collected and kept at −80°C until further assayed.

An oligonucleotide, 5′-TTCCGGC**TGACTCAT**-CAAGCG-3′, including the AP-1 motif (bold) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). To study AP-1 DNA binding, incubations were performed on ice for 30 min in a 20-μl reaction mixture containing 8 μg protein, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5% glycerol, 1 mM $MgCl_2$, 5 mM dithiothreitol, 0.1% bovine serum albumin, 1 μg/ml poly [dI–dC]–[dI–dC] and 0.2 ng of the double-stranded ^{32}P-labelled oligonucleotide probe. The specificity of the binding was determined in the presence of excess amounts of the unlabelled oligonucleotide. DNA-protein complexes were separated from the unbound probe on a native 5% polyacrylamide

gel in TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA). After electrophoresis gels were fixed, dried and autoradiographed. Autoradiograms were scanned and analysed densitometrically.

2.5. Antibodies and supershift analysis

Commercially available antibodies (Santa Cruz Biotechnology) against proteins of the AP-1 family, c-Fos (sc-52X), Fos B (sc-48X), c-Jun (sc-45X), Jun B (sc-46X) and Jun D (sc-74X), were used for the supershift analysis. They were supplied as affinity-purified polyclonal rabbit antibodies (1 μg immunoglobulin G per 1 μl in 0.05 sodium phosphate buffer) recognizing the specified rat antigens with no detectable cross-reactivity with other members of the Fos and Jun families. Nuclear extract protein (2 μg) prepared from cells of relevant treatment groups were incubated with 1 μg antibody in 10 μl of reaction mixture for 1 h on ice followed by the EMSA as described above.

3. Results

3.1. Effects of acute ethanol on carbachol-induced AP-1 DNA binding

As shown in Fig. 1, there was a certain level of basal AP-1 binding activity even in the absence of added muscarinic receptor agonist. The increased baseline AP-1 binding activity was reduced by the NMDA glutamate receptor antagonist, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohapten-5,10-imine maleate (MK-801) (data not shown here, but see Hou et al., 1997), suggesting that a constitutive, presumably endogenous glutamate-induced activation of NMDA receptors could be responsible. There were slight variations in the baseline AP-1 expression between different culture preparations probably depending on fluctuations in the concentration of glutamate released into the cell culture medium. Carbachol (100 or 300 μM, in the presence of 10 μM MK-801) enhanced the AP-1 binding by about 200% or 300%, as compared to the baseline level ($P < 0.005$ and $P < 0.001$ for 100 and 300 μM carbachol, respectively; Bonferroni's test of multiple comparisons). Various concentrations of ethanol (25–100 mM; Fig. 1) were applied together with carbachol (100 or 300 μM), but had no effect on the carbachol-induced AP-1 DNA binding.

3.2. Effects of chronic ethanol exposure on carbachol-induced AP-1 binding

As shown in Fig. 2, the carbachol-induced dose-dependent stimulation of AP-1 DNA binding ($F(5, 12) = 182$; $P < 0.0001$; two-way analysis of variance (ANOVA)) was significantly increased following 72 h ethanol exposure ($F(1, 12) = 38$; $P < 0.0001$; two-way ANOVA). Chronic ethanol exposure increased the potency of carbachol-produced AP-1 DNA binding by about two-fold (EC_{50} values were 57.1 ± 1.2 and 22.5 ± 1.3 μM for control and chronic ethanol-treated cells, respectively), whereas maximum stimulation by carbachol increased only marginally (20.1 vs. 21.7 band intensity units in control and chronic ethanol cultures, respectively). The baseline activity of AP-1 binding was not changed by chronic ethanol.

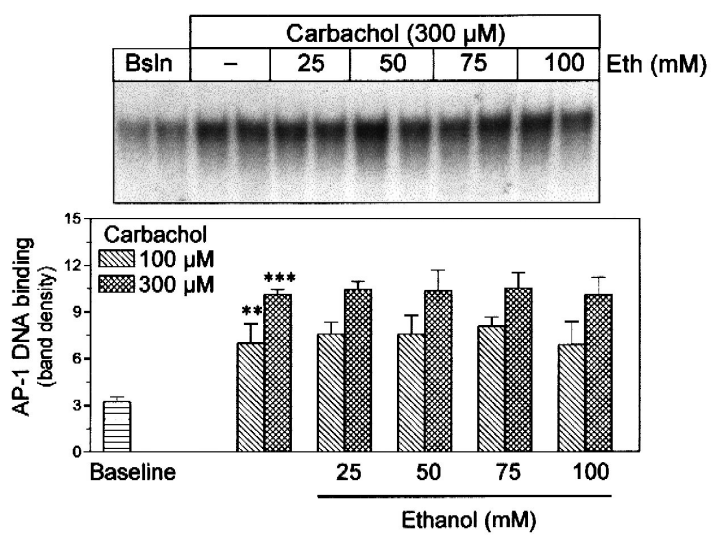

Fig. 1. Effect of acute ethanol application on carbachol-induced AP-1 DNA binding activity. Carbachol (100 or 300 μM) and/or ethanol at concentrations indicated was added directly to the culture medium and cultures were incubated for 2 h in CO_2 thermostat without a medium change. $^{***}P < 0.001$; $^{**}P < 0.005$ as compared to control cells. The data-points are means ± S.E.M. of two experiments from different cell culture preparations. Representative autoradiogram from cells treated with 300 μM carbachol is shown at the upper part of the figure.

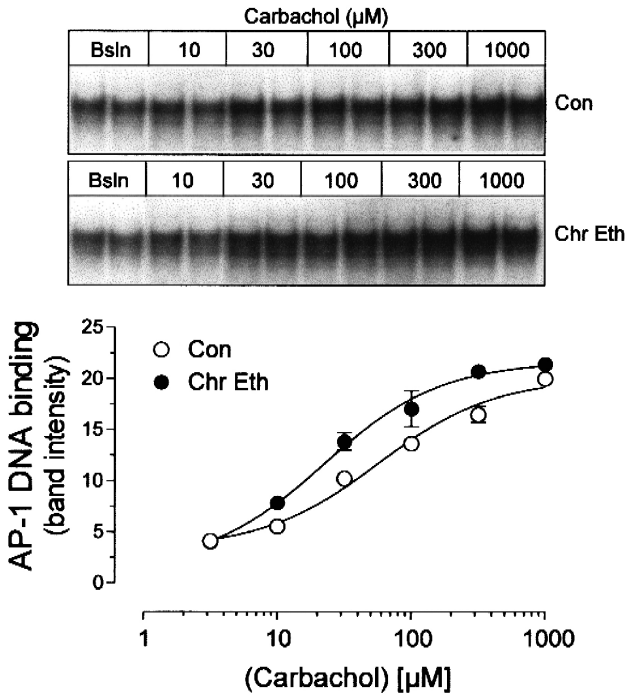

Fig. 2. Chronic ethanol potentiation of carbachol-induced AP-1 DNA binding activity. 'Chronic ethanol' cultures were exposed to rising concentrations of ethanol (from 50 to 100 mM) for 72 h. Carbachol was added to the ethanol-free medium (see Materials and methods) at the concentrations indicated. The data-points are the means ± S.E.M. of two experiments from different cell culture preparations. Statistical analysis appears in the Results section. Representative autoradiograms are shown in the upper part of the figure.

3.3. Protein composition of AP-1 complexes

The AP-1 transcription factor is known to be a dimer of c-Fos and c-Jun proteins or their cognates (Morgan and Curran, 1991). In order to study the protein composition of the AP-1 complex in our cultures, we analysed the AP-1 DNA-binding activity in the presence of antibodies against c-Fos, c-Jun, Fos B, Jun B or Jun D. In the absence of muscarinic receptor stimulation (*baseline*, Fig. 3), only Jun D was detected in the AP-1 complex. The carbachol-activated (100 μM) AP-1 complex contained Jun D and c-Fos proteins. The pattern of AP-1 protein expression induced by carbachol in cell cultures chronically exposed to ethanol was similar to that seen in the control cultures (Fig. 3). No supershifts were observed using anti-c-Jun, anti-Fos B, or anti-Jun B in either control or chronic ethanol cultures.

4. Discussion

Here we show that acute application of ethanol (25–100 mM) did not block muscarinic receptor-mediated carbachol-induced activation of the AP-1 transcription factor, whereas chronic ethanol exposure (50–100 mM ethanol during 3 days) produced a marked enhancement of the carbachol-induced AP-1 activation.

We have previously reported that carbachol-induced AP-1 activation is concentration-dependently inhibited by the muscarinic receptor subtype-specific antagonists pirenzepine and 1,1-dimethyl-4-diphenylacetoxypiperidinium iodite (4-DAMP), but not by gallamine, indicating that the carbachol induced AP-1 DNA binding is mediated predominantly via muscarinic M_3 and to a lesser extent M_1 (because pirenzepine was only effective at higher concentrations, > 1 μM, that can inhibit both M_1 and M_3 receptors), but not M_2 receptors (Cebers et al., 1999). Muscarinic M_1 and M_3 receptors share the same signal transduction mechanism involving activation of phospholipase C and subsequent formation of diacylglycerol and inositol 1,4,5-triphosphate (Hosey, 1992; Caulfield, 1993). Stimulation of this pathway induces activation of protein kinase C and mobilization of Ca^{2+} from intracellular stores (Felder, 1995). Protein kinase C activation plays an important role in the induction of c-*fos* proto-oncogene and activation of AP-1 transcription factor upon muscarinic receptor stimulation (Greenberg et al., 1986; Blackshear et al., 1987; Karin and Smeal, 1992; Hughes and Dragunow, 1993; Cohen et al., 1996). It has previously been demonstrated that acute application of ethanol in-

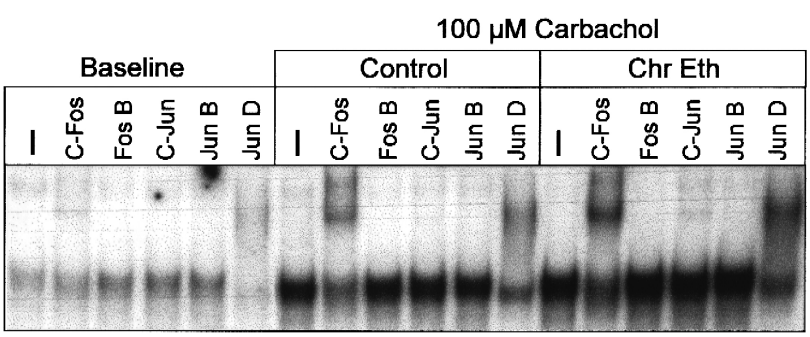

Fig. 3. Protein composition of AP-1 complexes was analysed using supershift assay with antibodies against peptide epitopes of c-Fos, Fos B, c-Jun, Jun B, Jun D in extracts prepared from control and chronic ethanol-treated cells exposed to acute carbachol (100 μM; 2 h). AP-1 band supershifts are observed with anti-c-Fos and anti-Jun D antibodies. Shown is an autoradiogram representative of similar results obtained in four different culture preparations.

hibits a number of muscarinic receptor-mediated intracellular events including activation of phospholipase C (Smith et al., 1986; Sanna et al., 1994). In contrast to those findings we were, however, unable to find any effect of acute application of ethanol (25–100 mM) on the carbachol-produced (100 or 300 μM) enhancement of AP-1 DNA binding. We have previously shown that the same concentrations of ethanol inhibit the stimulation of AP-1 DNA binding produced by the glutamate receptor agonist, NMDA (Cebers et al., 1996). Therefore, it can be concluded that NMDA and carbachol enhance the AP-1 DNA binding via activation of discrete signal transduction pathways that display differential sensitivity to acute ethanol. Furthermore, as carbachol has been shown to increase the free cytoplasmic Ca^{2+} concentration in cerebellar granule cells (Simpson et al., 1996), it appears that the carbachol-induced release of Ca^{2+} is not sensitive to acute ethanol application or, alternatively, Ca^{2+} release is not important for the carbachol-produced AP-1 DNA binding.

It has been reported that chronic ethanol exposure causes an up-regulation of the number of muscarinic binding sites and potentiates the effects of muscarinic receptor agonists (Hoffman et al., 1986; Pietrzak et al., 1989, 1990; Kim et al., 1993; Larson et al., 1996), as well as increases in the amount of mRNA encoding for the muscarinic receptors (Hu et al., 1993). Also, long-term ethanol exposure has been reported to enhance muscarinic M_1 receptor-mediated expression of c-*fos* and DNA binding of AP-1 in neuroblastoma SH-SY5Y cells (Ding et al., 1997). In concordance with the reports showing increased muscarinic receptor activity, we found that chronic ethanol exposure enhanced the carbachol-produced stimulation of AP-1 DNA binding. At present, we cannot indicate the cellular mechanisms involved in the enhanced responsiveness to carbachol in chronic ethanol-exposed cultures. One possibility is that chronic ethanol treatment increased the number and/or affinity of muscarinic receptors. Another explanation could be that, since carbachol may also induce release of Ca^{2+} from intracellular stores via a secondary activation of metabotropic glutamate receptors (Llansola et al., 1998), such signals could be altered by the chronic ethanol exposure. However, further studies are needed to clarify the effects of chronic ethanol on signaling pathways activated by muscarinic receptors.

In order to act as a transcription factor at AP-1 DNA binding sites, the c-Fos protein must dimerise with one of the members of the Jun family, Jun D, Jun B or c-Jun (Morgan and Curran, 1991). Various c-Fos/Jun heterodimers possess different transactivating potential depending on the Jun protein involved (Angel and Karin, 1991). For instance, c-Fos/c-Jun complexes activate transcription of the target genes, c-Fos/Jun B complexes may suppress transcription (Chiu et al., 1989; Schutte et al., 1989), whereas c-Fos/Jun D heterodimers have different effects depending on the target gene (DeGroot et al., 1992). Consequently, dimerization patterns of Fos and Jun proteins induced by different stimuli may cause functionally distinct effects on gene regulation. Our data show that cerebellar granule cells constitutively express a low level of AP-1 complexes, comprising Jun D protein. This in good agreement with previous studies indicating permanent activation of the *jun D* promoter (Hope et al., 1994). In line with our previous results in the same culture preparation (Cebers et al., 1999), carbachol application induced expression of AP-1 complexes comprising Jun D and c-Fos proteins. Thus, our data indicate that the potentiation of the carbachol-induced enhancement of AP-1 DNA binding following chronic ethanol treatment cannot be explained as due to a change in the protein composition of the AP-1 complex.

In summary, carbachol-induced AP-1 transcription factor DNA binding in cultured cerebellar granule cells was not altered by acute application of ethanol but was significantly enhanced by chronic ethanol exposure. These results suggest that muscarinic receptor-mediated activation of AP-1 transcription factor may be involved in ethanol-induced adaptive changes of the nervous system.

Acknowledgements

This study was supported by the Swedish Medical Research Council (projects no. 7688 and 3766), The Swedish Foundation for Alcohol Research, Åke Wibergs Foundation, Hans and Loo Ostermans Foundation, Kapten Artur Eriksson Foundation, Sigurd and Elsa Goljes Foundation, The Orion-Pharma Neurology Fellowship, and funds from the Karolinska Institute.

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