The Role of Protein Kinase C in Cellular Tolerance to Ethanol*

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Imogen R. Coe‡‡, Lina Yao‡, Ivan Diamond¶¶‡‡**‡‡, and Adrienne S. Gordon¶¶‡‡**‡‡

From the ‡‡Ernest Gallo Clinic and Research Center and the ¶¶Department of Neurology, ¶¶**Department of Cellular and Molecular Pharmacology and **Neuroscience Program, University of California, San Francisco, California 94110

We have shown that ethanol inhibits uptake of adenosine by a specific nucleoside transporter in NG108-15 neuroblastoma x glioma cells and that cAMP-dependent protein kinase (PKA) activity is required for this inhibition. After chronic exposure to ethanol, adenosine uptake is no longer inhibited on rechallenge with ethanol, i.e. transport has become tolerant to ethanol. Here we show that protein kinase C (PKC) contributes to ethanol-induced tolerance of adenosine transport. Activation of PKC by phorbol esters in control cells results in an ethanol-tolerant phenotype, similar to that produced by chronic ethanol exposure. In addition, chronic exposure to ethanol increases the amounts of α, β, and ε PKC. However, reducing PKC activity by inhibition with chelerythrine during chronic exposure to ethanol or down-regulation by phorbol esters prevents the development of ethanol-induced tolerance of adenosine transport. By contrast, the inhibition of PKA activity produces tolerance to ethanol inhibition of adenosine uptake. When protein phosphatase inhibitors are present, inhibiting PKA activity has no effect on ethanol sensitivity of adenosine uptake, suggesting a role for protein phosphatases in the regulation of ethanol sensitivity of uptake. Taken together, our results suggest that PKA and PKC have opposing effects on the ethanol sensitivity of adenosine transport; PKA activity is required for ethanol sensitivity, and PKC activation produces tolerance. Based on these data, we propose that chronic ethanol exposure increases PKC activity, leading to the activation of a protein phosphatase (1 or 2A). This phosphatase then dephosphorylates a PKA-phosphorylated site, which is required for ethanol to inhibit adenosine uptake. Therefore, the sensitivity of adenosine transport to ethanol appears to be maintained by a balance of PKA and protein phosphatase activities, and PKC may regulate phosphatase activity.

We have presented evidence that the cAMP signaling system is important for regulating the response to ethanol in a cell culture model of tolerance. We found that ethanol acts on a specific nucleoside transporter in NG108-15 neuroblastoma x glioma cells (1), inhibiting adenosine uptake (2). In cells treated chronically with ethanol, however, adenosine uptake becomes insensitive when rechallenged with acute ethanol, and adenosine uptake is no longer inhibited by ethanol (2). The loss of ethanol sensitivity is an example of ethanol tolerance at a cellular level. Using this model system, we have investigated the molecular mechanisms that regulate tolerance to ethanol.

We have shown that ethanol inhibition of adenosine uptake requires cAMP-dependent protein kinase (PKA) activity; ethanol does not inhibit adenosine uptake in mutant cells lacking PKA activity (3). We also found that the ethanol sensitivity of transport in cells chronically exposed to ethanol can be restored by activating PKA (4). In addition, the inhibition of PKA in naive cells reproduces the ethanol-tolerant phenotype. This latter effect can be prevented by inhibiting protein phosphatase activity with okadaic acid (4), suggesting that protein phosphatase (1 or 2A) is also involved in regulating the ethanol sensitivity of adenosine uptake. Based on this earlier work, we concluded that ethanol sensitivity of adenosine uptake appears to be due to a balance of PKA and phosphatase activities.

Recent evidence also implicates protein kinase C (PKC) in cellular responses to ethanol. On acute exposure to ethanol, PKC activity increases in human lymphocytes (5) and epidermal keratinocytes (6). Moreover, chronic exposure to ethanol causes increased PKC activity in NG108–15 and PC12 cells (7). PKC also regulates the ethanol sensitivity of GABA<sub>A</sub> receptors (8–10), NMDA receptors (11), AMPA/kainate receptors (12), and 5HT<sub>1C</sub> and M<sub>1</mub> muscarinic receptors (13). We therefore investigated the role of PKC in the regulation of ethanol sensitivity of adenosine transport. We found that activation of PKC produces the tolerant phenotype in naive cells and that inhibition or down-regulation of PKC during chronic exposure to ethanol prevents the development of tolerance. Our results suggest that PKC activity is required for the development of tolerance of ethanol sensitivity during exposure to ethanol.

EXPERIMENTAL PROCEDURES

Materials—The a and b isomers of phorbol 12-myristate 13-acetate (PMA) (LC Services, Woburn, MA) were dissolved in dimethyl sulfoxide or 95% ethanol to a concentration of 10 mM, stored at −20 °C, and diluted in assay medium (Dulbecco's modified Eagle's medium: F-12 (Ham's), 3:1; 25 mM HEPES, pH 7.4) to 100 nM for use in acute ethanol experiments. PMA was diluted in defined medium for chronic ethanol experiments. Bradykinin (Calbiochem) was dissolved in water to a concentration of 5 mM and diluted in assay medium to a concentration of 50 mM. Chelerythrine (Calbiochem) was dissolved in dimethyl sulfoxide to a concentration of 13 mM, stored at −20 °C, and diluted in defined medium to 1 mM. Nitrobenzylthioinosine, [H]adenosine (30 Ci/mmol), and non-radioactive adenosine were from Sigma, DuPont NEN, and Boehringer Mannheim, respectively. [3H]I-protein A was purchased from Amersham Corp., and anti-PKC isozyme-specific antibodies were from Santa Cruz Biotechnology, Inc.

Cell Culture—NG108-15 neuroblastoma x glioma hybrid cells (passage number 19–23) obtained from the cell culture facility at the University of California, San Francisco, were grown in 10% Nu-Serum and maintained for 2–3 days in complete defined medium as described.

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‡ Present address: Dept. of Biochemistry, 4-74 Medical Sciences Bldg., University of Alberta, Edmonton, Alberta, T6G2H7, Canada.

¶§ To whom correspondence should be addressed: Ernest Gallo Clinic and Research Center, SFGH Bldg. 1, Rm. 101, San Francisco, CA 94110. Tel.: 415-648-7111; Fax: 415-648-7116.

† The abbreviations used are: PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ANOVA, analysis of variance.
**RESULTS**

**Activation of PKC Causes Tolerance of Adenosine Transport in Control Cells**—Acute ethanol inhibits adenosine uptake by approximately 40% (Ref. 2 and Fig. 1). After chronic exposure to ethanol, adenosine uptake becomes tolerant to acute rechallenge with ethanol (Ref. 2 and Fig. 1). If increased amount and/or activity of PKC contributes to chronic ethanol-induced tolerance in NG108-15 cells, then activating PKC in naive control cells (cells not exposed to ethanol) should also produce ethanol tolerance. We therefore incubated naive cells for 10 min in the absence and presence of the phorbol ester, β-PMA (100 nM), which activates PKC, and then measured ethanol inhibition of adenosine uptake. Control cells showed a typical level of inhibition of adenosine uptake by acute ethanol (34 ± 6%, Fig. 1). By contrast, ethanol did not significantly inhibit adenosine uptake in cells pretreated with β-PMA. This loss of ethanol sensitivity of adenosine transport was similar to the tolerance caused by chronic ethanol exposure (Fig. 1). β-PMA had no further effect on the tolerance of adenosine uptake in cells treated chronically with ethanol (Fig. 1). In both naive cells and cells chronically exposed to ethanol, the absolute values of uptake in the absence of acute ethanol were not affected by β-PMA treatment (not shown). The inactive phorbol ester α-PMA had no effect on the ethanol sensitivity of adenosine uptake in control cells (Fig. 1), suggesting that the ethanol tolerance produced by β-PMA was due to the activation of PKC.

We also determined whether the activation of PKC by an endogenous receptor pathway produces the tolerant phenotype in naive cells. In NG108-15 cells, bradykinin receptors activate phospholipase C (17), which consequently activates PKC. Therefore, cells were incubated with bradykinin (50 μM) for 10 min before measuring ethanol sensitivity of adenosine uptake. Cells treated with bradykinin showed the tolerant phenotype; acute ethanol did not inhibit adenosine uptake (Fig. 2). Similar to the effect of β-PMA, incubation with bradykinin had no effect on the ethanol sensitivity of adenosine uptake in control cells (Fig. 1). These data suggest that activation of PKC induces tolerance to ethanol inhibition of adenosine transport in naive cells not previously exposed to ethanol.

**Chronic Ethanol Exposure Increases the Amount of Specific PKC Isozymes**—Since activation of PKC and chronic exposure to ethanol produced the same tolerant phenotype, it was possible that ethanol increased the amount of PKC in NG108-15 cells, leading to tolerance of adenosine transport. We therefore determined whether chronic exposure to ethanol alters the amount of PKC in NG108-15 cells grown in defined medium.
PKC is a family of closely related isozymes divided into three subfamilies (18): the Ca\(^{2+}\)-dependent or conventional isozymes (PKC\(\alpha\), \(\beta_1\), \(\beta_2\), \(\gamma\)), the Ca\(^{2+}\)-independent or novel isozymes (\(\delta\), \(\epsilon\), \(\zeta\), \(\iota\), and \(\mu\)), and phorbol ester-insensitive or atypical subfamilies (PKC\(\zeta\) and \(\iota/\lambda\)). Using isozyme-specific antibodies against the phorbol ester-sensitive PKC isozymes, we found that \(\alpha\), \(\beta_1\), \(\beta_2\), \(\delta\), and \(\epsilon\) PKC are expressed in these cells (Fig. 3A, control). Antibodies against \(\eta\), \(\theta\), and \(\mu\) were not available to us. Chronic exposure to 200 mM ethanol for 48 h caused a significant increase in the levels of PKC \(\alpha\), \(\delta\), and \(\epsilon\) when compared with naive control cells (Fig. 3A and B). These experiments were carried out at a relatively high concentration of ethanol to allow us to use short exposure times (2 days). To determine whether lower concentrations of ethanol also increase \(\delta\) and \(\epsilon\) PKC levels, NG108-15 cells were incubated in varying concentrations of ethanol for 4 days. The data in Fig. 3C indicate that the amounts of \(\delta\) and \(\epsilon\) PKC were significantly increased at 25 mM ethanol and that the increase was dependent on the concentration of ethanol.

PKC Inhibition during Chronic Ethanol Exposure Prevents the Development of Tolerance—If the increased amount of \(\alpha\), \(\delta\), or \(\epsilon\) PKC produced by chronic ethanol exposure contributes to the development of tolerance, then inhibiting PKC activity during ethanol treatment should prevent the development of the chronic ethanol phenotype. We used the specific PKC inhibitor, chelerythrine (19), to test this hypothesis. Cells were pretreated for 30 min either with or without 1 \(\mu\)M chelerythrine (Fig. 4). There was no significant change in the amount of the \(\beta_1\) and \(\beta_2\) isozymes (Fig. 3A and B). These experiments were carried out at a relatively high concentration of ethanol to allow us to use short exposure times (2 days). To determine whether lower concentrations of ethanol also increase \(\delta\) and \(\epsilon\) PKC levels, NG108-15 cells were incubated in varying concentrations of ethanol for 4 days. The data in Fig. 3C indicate that the amounts of \(\delta\) and \(\epsilon\) PKC were significantly increased at 25 mM ethanol and that the increase was dependent on the concentration of ethanol.

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ethanol for 48 h just as in untreated cells (Fig. 4). Chelerythrine had no effect on adenosine uptake in the absence or presence of ethanol in naive control cells. However, chelerythrine completely blocked the chronic ethanol-induced increase in the amounts of δ and ε PKC (Fig. 3, D and E).

Down-regulation of PKC Prevents the Development of Tolerance—The functional consequences of reduced PKC activity can also be determined by down-regulating PKC. Therefore, the PMA-sensitive PKC isozymes were down-regulated with β-PMA (20) prior to and during exposure to ethanol. Cells were pretreated for 30 min with medium alone or medium containing 100 nM β-PMA and then incubated in the presence or absence of ethanol, with or without 100 nM β-PMA for 48 h. The α, β₁, β₂, δ, and ε isozymes were virtually absent after β-PMA treatment (Fig. 3A). Chronic ethanol exposure did not produce the tolerant phenotype in these down-regulated cells; ethanol inhibition of adenosine uptake was similar in down-regulated ethanol-exposed cells and naive control cells (Fig. 5). Down-regulation of PKC had no effect on ethanol sensitivity of adenosine uptake in naive cells. Therefore, reduced PKC activity produced either by PKC inhibition (with chelerythrine) or down-regulation (by β-PMA) during chronic ethanol exposure prevented the development of the ethanol tolerant phenotype. These data support the hypothesis that an ethanol-induced increase in PKC amount or activity is required for tolerance of adenosine transport on rechallenge with ethanol.

DISCUSSION

We have shown that a specific facilitative nucleoside transporter is a primary target for ethanol in NG108-15 cells (1). Acute ethanol inhibition of adenosine uptake via this transporter leads to activation of adenosine A₂ receptors and a cascade of intracellular signaling events leading to tolerance of adenosine transport on rechallenge with ethanol (2). We found that adenosine uptake is not inhibited by ethanol in mutant cells lacking PKA activity (3) and that the tolerance of adenosine transport induced by chronic ethanol exposure could be reversed by activating PKA (4). These results suggest that PKA activity is required for ethanol to inhibit adenosine uptake. Treatment with phorbol esters (Fig. 1), which activate PKC, or activation of bradykinin receptors (Fig. 2), which also leads to activation of PKC (21, 22), produces a tolerant phenotype similar to that produced by chronic exposure to ethanol. Conversely, ethanol tolerance is prevented in chronically exposed cells when PKC activity is inhibited (Fig. 4) or reduced by down-regulation (Fig. 5).

These data suggest that the ethanol sensitivity of adenosine transport is regulated by both PKA and PKC. PKA maintains the ethanol-sensitive phenotype; PKC activity is required for chronic ethanol-induced tolerance of adenosine transport. There is evidence in other systems that a balance of PKA and PKC activities regulates cellular functions. PKA can overcome PKC-dependent desensitization of the G-protein-activated K⁺ channel (23), and opposing effects of PKA and PKC on the glycine receptor have also been reported (24). Hoek and coworkers (25) have also shown that PKA and PKC have opposite effects on the regulation of basal and receptor-stimulated phospholipase C activity by ethanol in liver.

In naive NG108-15 cells, activation of PKC produces the tolerant phenotype of adenosine transport (Fig. 1), but ethanol-
induced tolerance can be reversed by activating PKA. These observations are best explained by an indirect mechanism of action for PKC with protein phosphatase(s) 1 and/or 2A as intermediates (Fig. 6). We propose that PKC activates a phosphatase that dephosphorylates the PKA-phosphorylated site on the transporter or an associated regulatory protein producing the ethanol-insensitive phenotype. In support of this model, we found that inhibition of PKA activity produces an ethanol-tolerant phenotype only if protein phosphatase(s) 1 and/or 2A are active (4); in the presence of phosphatase inhibitors, inhibiting PKA activity had no effect on ethanol sensitivity of transport. Therefore, ethanol sensitivity of transport appears to be due to a balance between phosphorylation and dephosphorylation. In naive cells, phosphorylation predominates. Phosphatase activity predominates after inhibition of PKA activity or after chronic ethanol exposure. Phosphatase activity could be stimulated directly by PKC as described for the receptor-like protein-tyrosine phosphatase RPTPα (26) or indirectly by phosphorylation and inactivation of a phosphatase inhibitor protein as described for β-PMA activation of protein phosphatase 1 in L6 cells (27). Inhibition of PKC by chelerythrine or down-regulation of PKC by PMA in cells exposed chronically to ethanol would be expected to prevent activation of the phosphatase by PKC, leaving the PKA-phosphorylated site intact and resulting in an ethanol-sensitive phenotype.

PKC is a family of isozymes of which at least five are expressed in NG108-15 cells (Fig. 3A). Beckmann et al. (28) did not detect β1 in NG108-15 cells. This may reflect the difference in culture medium in which the cells are grown. We found that the amounts of α, δ, and ε were increased after chronic exposure to ethanol (Fig. 3B). Although we have not measured PKC activity directly, an increase in PKC activity correlated with increased amounts of α, δ, and ε PKC after chronic exposure of PC12 cells to ethanol (7). Since chelerythrine inhibited both PKC activity and the ethanol-induced increase in amounts of δ and ε PKC, it remains to be determined whether increases in PKC amount or PKC activity, per se, are required for the development of tolerance of adenosine transport.

In addition to changes in the amount or activity of PKA, PKC, or possibly protein phosphatases 1 and 2A, ethanol might alter the intracellular localization of these enzymes, thus altering their function. Recent evidence suggests that the localization of intracellular signaling enzymes determines their specificity of action (29–31). PKA (32), protein phosphatases 1 and 2A (30), and specific isozymes of PKC (33) have unique locations within a number of cell lines including NG108-15 cells (28). Ethanol has been shown to cause translocation of PKC activity from cytosolic to membrane fractions in astroglial cells (34), human lymphocytes (5), and epidermal keratinocytes (6). It is therefore possible that ethanol may modulate the balance of PKA, PKC, and protein phosphatase activities by altering their localization in NG108-15 cells. Such changes may contribute to the development of ethanol tolerance.

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REFERENCES
1. Krauss, S. W., Ghirnikar, R. B., Diamond, I., and Gordon, A. S. (1993) Mol. Pharmacol. 44, 1021–1026
2. Nagy, L. E., Diamond, I., Casso, D. J., Franklin, C., and Gordon, A. S. (1990) J. Biol. Chem. 265, 1946–1951
3. Nagy, L. E., Diamond, I., and Gordon, A. S. (1991) Mol. Pharmacol. 40, 812–817
4. Coe, I. R., Dohrmann, D. P., Constantinescu, A., Diamond, I., and Gordon, A. S. (1996) J. Pharmacol. Exp. Ther. 276, 365–369
5. DePetris, P. B., and Liou, C. S. (1993) Alcohol Clin. Exp. Res. 17, 351–354
6. Kharbanda, S., Nakamura, T., and Kufe, D. (1993) Biochem. Pharmacol. 45, 675–681
7. Messing, R. O., Petersen, P. J., and Henrich, C. J. (1991) J. Biol. Chem. 266, 23428–23432
8. Wafford, K. A., and Whiting, P. J. (1992) FEBS Lett. 313, 113–117
9. Weiner, J. L., Zhang, L., and Carlen, P. L. (1994) J. Pharmacol. Exp. Ther. 268, 1368–1375
10. Harris, R. A., McQuilkin, S. J., Paylor, R., Abeliovich, A., Tonegawa, S., and Wehner, J. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3658–3662
11. Snell, L. D., Iorio, K. R., Tabakoff, B., and Hoffman, P. L. (1994) J. Neurochem. 62, 1783–1789
12. Dildy-Mayfield, J. E., and Harris, R. A. (1995) J. Neurosci. 15, 3162–3171
13. Sanna, E., Dildy-Mayfield, J. E., and Harris, R. A. (1994) Mol. Pharmacol. 45, 1004–1012
14. Gordon, A. S., Collier, K., and Diamond, I. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2105–2108
15. Bradford, M. (1976) Anal. Biochem. 72, 248–254
16. Laemmli, U. K. (1970) Nature 227, 680 – 685
17. Wolsing, D. H., and Rosenbaum, J. S. (1991) J. Pharmacol. Exp. Ther. 257, 621–633
18. Nishizuka, Y. (1992) Science 258, 607–614
19. Herbert, J. M., Augereau, J. M., Gleye, J., and Maffrand, J. P. (1990) Biochem. Biophys. Res. Commun. 172, 993–999
20. Blackshear, P. J. (1988) Am. J. Med. Sci. 296, 231–240
21. Tippner, S., Quitterer, U., Kolm, V., Fausnner, A., Roscher, A., Mostlaf, L., Müller-Esterl, W., and Haring, H. (1994) Eur. J. Biochem. 225, 297–304
22. Gerwins, P., and Fredholm, B. B. (1995) Naunyn-Schmiedebergs Arch. Pharmacol. 351, 186–193
23. Chen, Y., and Yu, L. (1994) J. Biol. Chem. 269, 7839–7842
24. Vaele, M.-L., Ruiz-Gomez, A., Lerma, J., and Mayor, F., Jr. (1994) J. Biol. Chem. 269, 2002–2008
25. Hoek, J. B., Nomura, T., and Higashi, K. (1993) Alcohol Cell Membranes, and Signal Transduction in Brain (Alling, C., Diamond, I., Leslie, S. W., Sun, G. Y., and Wood, W. G., eds), pp. 219–234, Plenum Press, New York
26. Tracy, S., van der Geer, P., and Hunter, T. (1995) J. Biol. Chem. 270, 10587–10594
27. Sririvasan, M., and Begum, N. (1994) J. Biol. Chem. 269, 16662–16667
28. Beckmann, R., Lindschau, C., Haller, H., Hudho, F., and Buchner, K. (1994) Eur. J. Biochem. 222, 335–343
29. Mochly-Rosen, D. (1995) Science 268, 247–251
30. Hubbard, M. J., and Cohen, P. (1993) Trends Biochem. Sci. 18, 172–177
31. Inagaki, N., Ito, M., Nakano, T., and Inagaki, M. (1994) Trends Biochem. Sci. 19, 448–452
32. Scott, J. D., and McCartney, S. (1994) Mol. Endocrinol. 8, 5–11
33. Disatnik, M.-H., Buraggi, G., and Mochly-Rosen, D. (1994) Exp. Cell Res. 210, 287–297
34. Sleight, S., and Shain, W. (1990) Life Sci. 47, 1037–1042