Protein Kinase Cε Mediates Ethanol-induced Up-regulation of L-type Calcium Channels*

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Brief ethanol exposure inhibits L-type, voltage-gated calcium channels in neural cells, whereas chronic exposure increases the number of functional channels. In PC12 cells, this adaptive response is mediated by protein kinase C (PKC), but the PKC isozyme responsible is unknown. Since chronic ethanol exposure increases expression of PKCδ and PKCe, we investigated the role these isozymes play in up-regulation of L-type channels by ethanol. Incubation with the PKC inhibitor GF 109203X or expression of a PKCδ fragment that inhibits phorbol-ester-induced PKCδ translocation largely prevented ethanol-induced increases in dihydropyridine binding and K⁺-stimulated 45Ca²⁺ uptake. A corresponding PKCe fragment had no effect on this response. These findings indicate that PKCδ mediates up-regulation of L-type channels by ethanol. Remaining responses to ethanol in cells expressing the PKCδ fragment were not inhibited by GF 109203X, indicating that PKCδ-independent mechanisms also contribute. PKCδ overexpression increased binding sites for dihydropyridine and L-channel antagonists, but did not increase K⁺-stimulated 45Ca²⁺ uptake, possibly because of homeostatic responses that maintain baseline levels of channel function. Since L-type channels modulate drinking behavior and contribute to neuronal hyperexcitability during alcohol withdrawal, these findings suggest an important role for PKCδ in alcohol consumption and dependence.

Understanding biochemical mechanisms that underlie alcohol tolerance and dependence may lead to new treatments for alcoholism. In nonalcoholic persons, intoxication develops at blood alcohol levels of 10–35 mM, and acute tolerance develops rapidly, so that after a few hours, an individual can appear sober at alcohol levels that previously caused intoxication (1). The mechanism for acute tolerance may involve activation of Fyn kinase and reduced sensitivity of tyrosine-phosphorylated N-methyl-d-aspartate receptors to inhibition by alcohol (2). Chronic tolerance is characteristic of alcoholicism, and its magnitude in alcoholics can be quite striking. For example, blood alcohol concentrations above 100 mM produce coma in a nonalcoholic person, whereas human alcoholics may appear sober or only mildly intoxicated with blood levels of 100–150 mM (3, 4).

The ability of ethanol to alter the function of neuronal voltage-dependent calcium channels appears to contribute to chronic tolerance. In several neural preparations, ethanol inhibits voltage-dependent calcium influx and calcium currents (5–13). Chronic exposure results in the development of tolerance to the inhibitory actions of ethanol on calcium channels (6, 7). The mechanisms underlying this adaptive response have been studied most in the neural cell line PC12. In PC12 cells, prolonged exposure to 25–200 mM ethanol for 2–6 days produces a reversible concentration- and time-dependent increase in K⁺-evoked 45Ca²⁺ uptake (8, 9, 14) and L-type calcium currents (14) measured in the absence of ethanol. This is associated with a corresponding increase in the number of binding sites for dihydropyridine Ca²⁺ channel antagonists (8, 9), suggesting that cells adapt to chronic ethanol exposure by increasing expression of functional L-type calcium channels. Similar increases in dihydropyridine binding have been detected in ethanol-treated NG108-15 neuroblastoma × glioma cells (15) and in brain membranes from ethanol-treated rats (16).

Up-regulation of L-type channels could promote alcohol consumption since L-channel antagonists reduce consumption in animals (17–20). Increases in L-type calcium channels may also contribute to the intense neuronal hyperexcitability observed during alcohol withdrawal (21). A role for L-type channels in alcohol withdrawal syndromes is supported by evidence that L-channel antagonists reduce tremors, seizures, and mortality in alcohol-dependent rodents deprived of ethanol (22–24). Moreover, ethanol-induced increases in binding sites for L-channel antagonists are greater in mice bred for severe alcohol withdrawal seizures than in mice bred for minor signs of alcohol withdrawal (25). These findings suggest that L-channel up-regulation plays an important role in alcohol dependence.

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases involved in cell growth and differentiation, neurotransmitter release and receptor regulation, ion channel modulation, and gene expression (26). Eleven PKC isoforms have been identified (α, βI, βII, γ, δ, ε, ζ, η, θ, λ, and μ), and they differ in structure and requirements for activation by diacylglycerol and calcium (26–28). In PC12 cells, we found that up-regulation of L-type channels by ethanol is inhibited by the kinase inhibitors sphingosine and polymyxin B (29). The effect of sphingosine is reversed by phorbol esters that activate all PKC isoforms except PKCζ and PKCλ (26), suggesting that ethanol-induced up-regulation of L-type channels requires activation of a phorbol-ester-sensitive PKC. We also found that chronic ethanol exposure increases total PKC activity, high affinity phorbol ester binding, and PKC-mediated phosphorylation in PC12 cells (30). This is associated with a selective increase in immunoreactivity (30) and mRNA (31) for two PKC isoforms, PKCδ and PKCe. Taken together, these findings

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The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ANOVA, analysis of variance.
suggest that chronic exposure to ethanol up-regulates functional L-type channels through a mechanism that involves ethanol-induced increases in expression of PKCδ or PKCe.

In this paper, we examined whether PKCδ or PKCe mediates ethanol-induced increases in L-type channels by using stably transfected PC12 cell lines that express the δV1 or eV1 fragment, which are derived from the first variable domains of PKCδ and PKCe, respectively. These fragments selectively inhibit phorbol ester-induced translocation of the corresponding isoforms, and the eV1 fragment specifically prevents phorbol ester-mediated inhibition of contraction in cultured cardiac myocytes (32) and enhancement of nerve growth factor-induced neurite outgrowth and mitogen-activated protein kinase activation by ethanol or phorbol esters in PC12 cells (33). We found that chronic exposure to ethanol increased K\(^+\)-stimulated \(^{45}\)Ca\(^{2+}\) uptake and dihydropyridine binding in PC12 cells, vector-transfected cells, and cells expressing eV1, but not in cells expressing δV1. We also found that overexpression of PKCδ increased dihydropyridine binding, but did not enhance K\(^+\)-stimulated \(^{45}\)Ca\(^{2+}\) uptake. These results demonstrate that PKCδ mediates increases in L-type calcium channels induced by chronic exposure to ethanol. These results are the first to demonstrate a functional role for PKCδ in neural cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—In these experiments, we used PC12 cells obtained from Dr. John A. Wagner (Cornell University, New York). Cells were grown at 37 °C in plastic tissue culture flasks in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 10% horse serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 90% air and 10% CO\(_2\). Cells were cultured with ethanol in tightly capped tissue culture flasks or in six-well plates wrapped in Parafilm, and the medium was changed daily as described previously (8, 29). Parallel control samples were cultured in a similar manner without ethanol. Stably transfected cell lines that overexpress PKCδ or PKCe have been described previously (34). Cell lines that express Flag epitope-tagged δV1 or eV1 were cultured and analyzed by reverse transcriptase-polymerase chain reaction and Western analysis for the Flag epitope tag as described (33).

**Phorbol Ester-stimulated Translocation of PKCδ—**Cells (4 x 10\(^6\)) were plated on 100-mm\(^2\) plastic tissue culture plates. After 2 days, the plates were rinsed with 10 ml of Dulbecco’s modified Eagle’s medium and incubated with or without 30 μM phorbol 12-myristate 13-acetate (PMA) for 2 min at 37 °C. Cells were rinsed twice with Ca\(^2+\)- and Mg\(^2+\)-free phosphate-buffered saline and scraped into ice-cold buffer containing 20 mM Tris-Cl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, 1 μg/ml phenylmethylsulfonyl fluoride. Cells were homogenized in a Teflon-glass homogenizer, and sucrose was added to a final concentration of 250 mM. Samples were then centrifuged at 150,000 x g for 1 h, and the supernatant was saved as the cytosolic fraction. The pellet was dispersed by sonication, and samples of supernatant and pellet derived from 100 μg of crude homogenate were analyzed by Western analysis as described (33).

**K\(^+\)-stimulated \(^{45}\)Ca\(^{2+}\) Uptake**—PC12 cells were plated onto poly-lysine-coated six-well tissue culture plates at a density of 0.8–1.6 x 10\(^6\) cells/well. After 24 h, cells were cultured for another 1–6 days in the presence or absence of ethanol. On the day of assay, cells were rinsed twice with 1 ml of 5 mM KCl buffer (85 mM NaCl, 5 mM KCl, 45 mM choline chloride, 2 mM CaCl\(_2\), 5 mM glucose, and 25 mM HEPES, pH 7.4) at room temperature, and incubated in the same buffer for 25 min. Cells were then incubated at 25 °C in 5 or 50 mM KCl buffer containing 0.75 μCi of \(^{45}\)Ca\(^{2+}\). The composition of the 50 mM KCl buffer was identical to that of the 5 mM KCl buffer except that choline chloride was replaced by KCl. After incubation for 25 min, cells were washed four times with 2.5 ml of ice-cold 5 mM KCl buffer and incubated overnight with 1 ml of 1 M NaOH. Radioactivity in neutralized samples was measured by liquid scintillation counting, and protein levels were determined by the method of Lowry et al. (35). K\(^+\)-stimulated uptake is defined as the difference between uptake in 50 and 5 mM KCl buffers and represents 85 ± 1% (n = 10) of total uptake in 50 mM KCl buffer.

**RESULTS**

**GF 109203X Prevents Ethanol-induced Increases in K\(^+\)-stimulated \(^{45}\)Ca\(^{2+}\) Uptake**—Previous work suggested that up-regulation of L-type channels by ethanol requires activation of PKC since it is prevented by the nonspecific PKC inhibitors spingosine and polymyxin B (29). Since we had used a different clone of PC12 cells for these earlier studies, we needed to determine whether our current PC12 cell line responds similarly to ethanol. In control cells, \(^{45}\)Ca\(^{2+}\) uptake was 3.6 ± 0.21 nmol of Ca\(^{2+}\)/mg of protein/2.5 min (n = 103) and was inhibited 92 ± 2% by the L-type channel antagonist nimo- dipine from binding in its absence and represents 51 ± 2% (n = 10) of total binding at 50 μM radioisotid!

**Characterization of V1-expressing Cell Lines**—To investigate whether PKCδ or PKCe is required for up-regulation of L-type calcium channels by ethanol, we created PC12 cell lines that stably express V1 fragments derived from the first variable
domains of PKCδ (δV1) and PKCe (eV1). These fragments inhibit phorbol ester-induced translocation and activation of their corresponding PKC isozyme (32, 33). Characterization of eV1-expressing PC12 cell lines (V1e1 and V1e2) was described recently (33). Expression of the δV1 fragment in V1δ2, V1δ3, and V1δ4 cells was confirmed by reverse transcriptase-polymerase chain reaction (Fig. 2A) and Western analysis (Fig. 2B). Treatment with 30 nM PMA stimulated translocation of both PKCδ and PKCe to the particulate fraction in the parent PC12 cell line and in C cells transfected with vector alone (Fig. 2, C and D). However, in cell lines expressing the δV1 fragment, translocation of PKCδ was inhibited, whereas translocation of PKCe was not (Fig. 2, C and D). In contrast, PMA-induced translocation of PKCe is selectively inhibited in eV1-expressing lines (33). Thus, expression of δV1 or eV1 fragments selectively inhibits phorbol ester-stimulated translocation of the corresponding PKC isozyme in these cells.

The δV1 Fragment Prevents Ethanol-induced Increases in K⁺-stimulated 45Ca²⁺ Uptake—To determine whether PKCδ or PKCe mediates ethanol-induced increases in L-type channel function, we measured K⁺-stimulated 45Ca²⁺ uptake in cells expressing δV1 or eV1. Expressed as a percentage of K⁺-stimulated 45Ca²⁺ uptake measured in PC12 cells, uptake was similar (p = 0.11; ANOVA) in C (88 ± 6%, n = 23), V1δ2 (107 ± 10%, n = 15), V1δ3 (118 ± 7%, n = 6), V1δ4 (85 ± 6%, n = 8), V1e1 (101 ± 7%, n = 6), and V1e2 (98 ± 5%, n = 6) cells. Treatment with 150 mM ethanol for 6 days increased 45Ca²⁺ uptake in cells expressing the δV1 fragment (V1δ3, V1δ4, or V1δ2). The data shown are from a representative experiment. The data shown are the means ± S.E. (n = 6–24) and are expressed as the percent above 45Ca²⁺ uptake measured in parallel control cells cultured without ethanol. *p < 0.05 compared with PC12 or C cells (ANOVA and Newman-Keuls test).

The δV1 Fragment Prevents Ethanol-induced Increases in Dihydropyridine Binding—To determine if expression of the δV1 fragment also prevents ethanol-induced increases in dihydropyridine binding, we measured binding of the L-type calcium channel antagonist (+)-[3H]PN200-110 to ethanol-treated cells (36). For these studies, we selected the two δV1-expressing clones in which the response to ethanol was most inhibited in the 45Ca²⁺ uptake assay. Basal (+)-[3H]PN200-110 binding to PC12 cells was 3.52 ± 0.43 fmol/mg (n = 15) and was similar (p = 0.14; ANOVA) to binding measured in C (4.43 ± 0.42 fmol/mg, n = 7), V1δ2 (3.78 ± 0.64 fmol/mg, n = 8), V1δ3...
and V1

L-channel density rather than an increase in binding affinity in fmol/mg). These findings are consistent with an increase in examining dihydropyridine binding, \( K_1 \)

dexamines PC12 cells. *, S.E. from 3 to 19 experiments and are expressed as the percent of uptake and binding above or below that measured in parallel cultures of PC12 cells. *, \( p < 0.016 \) by one-sample t test.

\((4.85 \pm 0.49 \text{ fmol/mg, } n = 7)\), V1e1 (3.17 \pm 0.32 \text{ fmol/mg, } n = 8)\), and V1e2 (3.17 \pm 0.37 \text{ fmol/mg, } n = 6)\). Treatment with 150 mM ethanol for 6 days increased binding to a similar extent in PC12, C, V1e1, and V1e2 cells (Fig. 3B). In contrast, ethanol failed to increase binding in V1e2 and V1e3 cells. These results suggest that PKC\( \delta \) is required for up-regulation of L-type channels by ethanol.

Overexpression of PKC\( \delta \)—To examine whether increases in PKC\( \delta \) are sufficient to increase L-channel density, we examined (+)-[\( ^{3}H \)]PN200-110 binding in PC12 cell lines \( \delta 1 \) and \( \delta 2 \), which overexpress PKC\( \delta \) (34). Compared with nontransfected PC12 cells, binding of 50 pm (+)-[\( ^{3}H \)]PN200-110 was increased in \( \delta 1 \) and \( \delta 2 \) cells, but not in C cells transfected with vector alone (Fig. 4). To determine whether the increase in binding in PKC\( \delta \)-overexpressing cells was due to a change in binding affinity or binding site number, binding was measured in \( \delta 2 \) cells at increasing concentrations of (+)-[\( ^{3}H \)]PN200-110 (from 10 to 280 ps). Fig. 5 shows a representative experiment. Scatchard analysis yielded similar values for the equilibrium dissociation constant (\( K_D \)) in PC12 (84 \pm 4 pm) and \( \delta 2 \) (102 \pm 14 pm) cells (\( p = 0.27; n = 3 \)). In contrast, the maximal number of binding sites (\( B_{\text{max}} \)) in \( \delta 2 \) cells (16.8 \pm 1.9 fmol/mg) was greater (\( p < 0.028; n = 3 \)) than the \( B_{\text{max}} \) in PC12 cells (10.0 \pm 0.7 fmol/mg). These findings are consistent with an increase in L-channel density rather than an increase in binding affinity in cells that overexpress PKC\( \delta \). However, despite the increase in dihydropyridine binding, K*-stimulated [\( ^{46} \text{Ca}^{2+} \)] uptake was not increased in \( \delta 1 \) or \( \delta 2 \) cells (Fig. 4).

PKC\( \delta \)-independent Regulation of L-type Channels by Ethanol—Since overexpression of PKC\( \delta \) increased dihydropyridine binding but not K*-stimulated [\( ^{46} \text{Ca}^{2+} \)] uptake, we considered whether enhancement of L-channel function by ethanol requires additional, PKC\( \delta \)-independent mechanisms. Initial evidence for a PKC\( \delta \)-independent mechanism was obtained by examining SV1-expressing cells treated with ethanol and PKC inhibitors. Although treatment with GF 109203X substantially inhibited ethanol-induced increases in K*-stimulated [\( ^{46} \text{Ca}^{2+} \)] uptake in PC12 cells (Fig. 1A), in V1e4 cells, it did not prevent increases due to ethanol, which were 26 \pm 3% of control in the absence and 25 \pm 5% of control in the presence of GF 109203X (\( p = 0.9; n = 6 \)). This result indicates that a PKC\( \delta \)-independent mechanism activated by ethanol contributes to L-channel up-regulation. To examine if such a PKC\( \delta \)-independent mechanism is required to increase L-channel function in PKC\( \delta \)-overexpressing cells, we treated \( \delta 1 \) and \( \delta 2 \) cells with 150 mM ethanol for 5 days. We predicted that this treatment would markedly increase [\( ^{46} \text{Ca}^{2+} \)] uptake with little or no effect on dihydropyridine binding. Ethanol enhanced K*-stimulated [\( ^{46} \text{Ca}^{2+} \)] uptake in these cells (Fig. 6A) without increasing PKC\( \delta \) immunoreactivity (Fig. 6B), consistent with activation of a PKC\( \delta \)-independent mechanism. However, the increase in [\( ^{46} \text{Ca}^{2+} \)] uptake was modest and associated with a similar increase in dihydropyridine binding (Fig. 6A).

**DISCUSSION**

In this paper, we found that ethanol-induced increases in L-channel density and function were largely prevented by expression of \( \delta \)SV1, a selective inhibitor of PKC\( \delta \) translocation. In contrast, expression of \( \delta \)V1, which inhibits PKCe translocation, did not prevent L-channel up-regulation. Expression of \( \delta \)V1 did not alter L-channel density or function in the absence of ethanol. These results indicate that PKC\( \delta \), but not PKCe, is important for ethanol-induced increases in functional L-type calcium channels in PC12 cells, but not for the basal activity of these channels.

Since ethanol increases the abundance of PKC\( \delta \) (30), we examined whether overexpression of PKC\( \delta \) would mimic the effect of ethanol on L-channel density and function. Although PKC\( \delta \) overexpression increased the number of dihydropyridine-binding sites in PC12 cells, it did not increase K*-stimulated [\( ^{46} \text{Ca}^{2+} \)] uptake. This could have occurred because PKC\( \delta \)-independent mechanisms are also required to increase channel function. Two results provided evidence for PKC\( \delta \)-independent mechanisms that are activated by ethanol. In V1e4 cells, PKC\( \delta \) translocation was completely blocked with only partial sup-
PKCδ and L-channels

One mechanism by which ethanol, acting via PKCδ, could increase L-channel density is by increasing expression of channel subunits. Neuronal high voltage-activated calcium channels are multimeric complexes of at least three types of subunits: α1, α2δ, and β (44). The major pharmacological and physiological features that distinguish different classes of voltage-gated channels are mainly due to α subunits, which contain the calcium pore and binding sites for selective calcium channel antagonists. There are five genes known to encode α1 subunits in brain (α1A, α1B, α1C, α1D, and α1F), and α1A and α1D are subunits of L-type channels (45–48). PC12 cells express α1C (49). Transfected α1C can form functional L-type channels, and coexpression with α2δ or β subunits results in increased channel function and a corresponding increase in dihydropyridine binding (47, 50–54). Thus, ethanol-induced increases in abundance of α1A, α2δ, or β subunits could increase the number of functional L-type channels. This might occur at a transcriptional level since PKCδ activates AP-1-Jun-regulated gene expression (55, 56). Studies are currently underway to determine if chronic exposure to ethanol regulates expression of specific calcium channel subunits by a PKCδ-dependent mechanism.

PKCδ is ubiquitously expressed and has been implicated in control of cell growth (56–58), apoptosis (59), and exocytosis (60) in non-neuronal cells. Little is known about its role in neuronal cells. It is induced in rat brain through an N-methyl-D-aspartate receptor-dependent mechanism after transient focal ischemia (61), but its role in brain injury or repair is not known. PKCδ binds to the growth-associated protein GAP-43 and appears to act as a GAP-43 kinase (62). However, it is not yet clear if PKCδ specifically regulates functions such as neurite growth or neurotransmitter release, which appear to be modulated by GAP-43 (63, 64). Our results provide the first evidence of a functional role for PKCδ in neural cells. Our findings identify PKCδ as a regulator of L-channel density and a mediator of cellular adaptation to ethanol. Since L-type channels modulate drinking behavior (17–20) and contribute to manifestations of alcohol withdrawal (22–24), PKCδ may play a key role in alcohol consumption and dependence. Ongoing studies will determine if inhibition of PKCδ reduces ethanol consumption and the development of alcohol dependence in animals.

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