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Overexpression of protein kinase C-ε enhances the stimulatory effect of ethanol on phospholipase C-mediated hydrolysis of phosphatidylethanolamine in NIH 3T3 fibroblasts

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Previously, ethanol and the protein kinase C (PKC) activators phorbol 12-myristate 13-acetate (PMA) and bombesin were shown to synergistically stimulate phospholipase C (PLC)-mediated hydrolysis of phosphatidylethanolamine (PtdEtn) in NIH 3T3 fibroblasts. Here we used fibroblasts overexpressing PKC-ε 15-fold to examine the possible role of this enzyme in the regulation of PtdEtn hydrolysis by ethanol. Overexpressed PKC-ε (i) greatly enhanced the stimulatory effects of ethanol (37.5–150 mM) on PLC-mediated PtdEtn hydrolysis, and (ii) eliminated the need for the co-presence of a PKC activator for maximal (3.3-fold) stimulation of PLC by 150 mM ethanol. Results suggest that PKC-ε is a potential positive regulator of the PtdEtn-hydrolyzing PLC activity, and that the functional interaction between PKC-ε and PLC is facilitated by ethanol.

Ethanol; Phospholipase C; Protein kinase C

1. INTRODUCTION

Work in our laboratory has revealed two specific effects of ethanol on the hydrolysis of phosphatidylethanolamine (PtdEtn) in NIH 3T3 fibroblasts. In the presence of an activator of protein kinase C (PKC), such as phorbol 12-myristate 13-acetate (PMA), ethanol was found to rapidly, but only transiently, enhance phospholipase D-mediated hydrolysis of PtdEtn [1]. Because this co-stimulatory effect of ethanol is transient, presently we cannot assign any possible (patho)physiological role for this process. More importantly, ethanol also exerts a longer-term stimulatory effect on phospholipase (PLC)-mediated PtdEtn hydrolysis [2]. Although this latter ethanol effect starts only after a 20–30 min lag period, it lasts for several hours causing significant decreases in the cellular content of PtdEtn [2]. Since PtdEtn is a major phospholipid in the cytoplasmic leaflet of plasma membrane, a reduction in its cellular content by activated PLC may contribute to the mediation of biological effects of ethanol. Hence, our interest in this phenomenon.

Interestingly, while PMA alone inhibited PLC-mediated PtdEtn hydrolysis by a PKC-α-mediated process, it enhanced the stimulatory effect of ethanol on this PLC activity [2]. This would be consistent with positive regulation of PLC activity by another PKC isozyme different from PKC-α.

NIH 3T3 fibroblasts contain only the α-, ε-, and ζ-PKC isozymes in detectable amounts ([3]; and our unpublished observations). Since PKC-α negatively regulates PLC activity [2] and PKC-ζ does not interact with PMA [4–7], PKC-ε appeared to be the most likely candidate to mediate the synergistic stimulatory effects of ethanol and PMA on PLC-mediated PtdEtn hydrolysis. Here we examined this possibility in NIH 3T3 fibroblasts overexpressing PKC-ε about 15-fold. We show that overexpressed PKC-ε potentiates the stimulatory effect of ethanol on PLC-mediated PtdEtn hydrolysis.

2. EXPERIMENTAL

2.1. *Materials*

PMA, Dowex-50W(H^+ form), phenylmethylsulfonyl fluoride (PMSF), leupeptin, and aprotinin were purchased from Sigma; biotinylated goat anti-rabbit IgG, streptavidin-alkaline phosphatase conjugate, nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were bought from Boehringer-Mannheim. Polyclonal antibodies raised against the α-, ζ-, and ε-PKC isozymes were kindly donated by Dr. Y.A. Hannun (Duke University, Durham, NC, USA); [2-^{14}C]ethanolamine (50 mCi/mmol) was from Amersham; the pSPORT I plasmid, tissue-culture reagents, and the lipofection transfection kit were bought from Gibco BRL; the restriction endonucleases *Sma*I, *Ear*I, ALW NI, and *Bsm*I were bought from New England Biolabs; the restrictive endonucleases *Hin*dIII, ALW 26I, and *Cla*I were from Promega; the pcDNA 3 expression vector was from Invitrogene.

2.2. *Cell culture*

The NIH 3T3 clone-7 fibroblast line was provided by Dr. D.R. Lowy (National Cancer Institute, NIH, Bethesda, MD, USA). The fibroblasts were continuously cultured in Dulbecco's modified Eagle's

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Abbreviations: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PtdEtn, phosphatidylethanolamine; PLC, phospholipase C; EtnP, ethanolamine phosphate.

medium supplemented with 10% (v/v) fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and glutamine (2 mM).

2.3. *Generation of PKC-ε overexpressing cell line*

The PKC-ε/pTB 803 pLTR-based cDNA clone, expressing full-length rat PKC-ε [8], was a generous gift from Dr. Yositaka Ono (Kobe University, Kobe, Japan). The original pTB 803 plasmid was changed to contain the neomycin resistant gene derived from the pcDNA 3 expression vector. In this vector, the neomycin resistance gene is driven by the SV_{40} promotor–origo complex; using *Hin*dIII and *Sma*I, the SV_{40} complex was replaced by a bacterial promoter sequence present in pSPORT I plasmid by using the ALW 261 and *Ear*I restriction endonucleases. The neomycin resistance gene from this construct was cloned into the *Cla*I site of pTB 803 clone by using the ALW NI and *Bsm*I restriction endonucleases. NIH 3T3 fibroblasts were transfected with either empty vector as a control, or with the PKC-ε expression vector using lipofection, following the procedure recommended by the manufacturer (Gibco BRL). The transfected cells were subsequently grown for 10 days in complete Dulbecco's medium containing 900 μg/ml neomycin, and then for 16 days in the presence of 600 μg/ml neomycin. After 26 days in selection medium, single colonies were picked and examined for the presence of PKC-ε by Western blot analysis. Vector control cells (NIH 3T3/PKC-ε_1) and one clone overexpressing PKC-ε about 15-fold (NIH 3T3/PKC-ε_{15}) were used in most experiments to determine PLD-mediated hydrolysis of PtdEtn.

2.4. *Western Blot analysis of PKC isozymes*

Attached NIH 3T3 cells (~ 70–80% confluent) in 150-mm-diameter dishes were incubated in Dulbecco's medium in the absence or presence of 100 nM PMA and/or ethanol (150 mM) for 5 min. Thereafter, cells were scraped into 4-ml homogenization buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM sucrose, 1 mM PMSF, 100 μg/ml leupeptin and 25 μg/ml aprotinin. After homogenization, homogenates were first centrifuged at $800 \times g$ for 6 min, and then supernatants were centrifuged at $15{,}000 \times g$ for 20 min to prepare cytosolic and particulate fractions. Protein fractions (50 μg protein each) were subjected to 8% SDS/PAGE, transferred to Immobilon-P transfer membranes (Millipore), and membranes were processed to visualize PKC-ε as described earlier [2]. Molecular masses were estimated with biotinylated standard proteins (Bio-Rad). Relative amounts of PKC isoenzymes were estimated densitometrically, using a Hoefer GS-300 Scanning Densitometer equipped with GS-365 Data System and Hewlett Packard Vectra CS computer.

2.5. *Measurement of PLC-mediated hydrolysis of PtdEtn in intact NIH 3T3 fibroblasts*

This was performed using fibroblasts prelabeled with [^{14}C]ethanolamine for 48 h as described earlier [2]. [^{14}C]Ethanolamine phosphate (EtnP), the product of [^{14}C]PtdEtn hydrolysis by PLC, was separated from other products on Dowex-50W(H^+) columns as previously indicated [2].

3. RESULTS AND DISCUSSION

A Western blot analysis of PKC isozymes revealed that in the NIH 3T3 fibroblast line (clone-7) used here, the α-, ε-, and ζ-isozymes are present in detectable amounts, while PKC-δ is barely detectable, and the β_1-, β_2-, and γ-isozymes are not detectable at all (data not shown). In view of the inhibitory effect of PKC-α on PLC activity [2] and the inability of PKC-ζ to respond to PMA [4–7], we considered the possibility that the observed synergistic effects of ethanol and PMA on PLC-mediated PtdEtn hydrolysis [2] might involve PKC-ε. Since this PLC activity is associated with membranes [2], an additional assumption was that ethanol and PMA may synergistically enhance the membrane content of the regulatory PKC isozyme. For these reasons, we first examined the individual and combined effects of ethanol and PMA on membrane translocation of PKC-ε in normal NIH 3T3 (clone-7) fibroblasts.

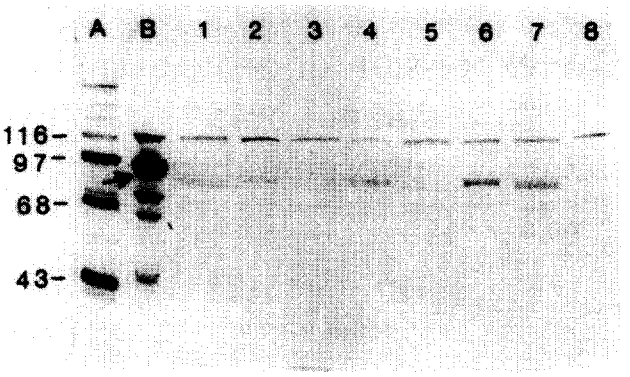

Fig. 1. Western blot analysis of PKC-ε in PMA- and ethanol-treated NIH 3T3 fibroblasts. Treatments of fibroblasts with 0.01% dimethyl sulfoxide (lanes 1,2), 100 nM PMA (lanes 3,4), PMA plus 150 mM ethanol (lanes 5,6), or ethanol alone (lanes 7,8) were for 5 min. Lanes 1,3,5,7 and 2,4,6,8 are soluble and particulate fractions, respectively. Lanes A and B are molecular size markers (indicated at the left side in kDa) and rat brain standards, respectively. The arrow indicates the position of PKC-ε.

In these fibroblasts, PKC-ε is about 5–8 times less abundant than PKC-α or PKC-ζ. In untreated fibroblasts, PKC-ε is almost equally distributed between the cytoplasmic (Fig. 1, lane 1) and membrane fractions (Figs. 1, lane 2). However, while PKC-ε in the cytosol corresponds to a non-phosphorylated form (faster-moving band), the membrane bound form (slower-moving band) can be labeled with $^{32}P_i$, i.e. it represents a phosphorylated form (data not shown). Treatment of fibroblasts with 100 nM PMA for 5 min essentially eliminated the cytoplasmic pool of PKC-ε (Fig. 1, lane 3) with some of this enzyme redistributing to the mem-

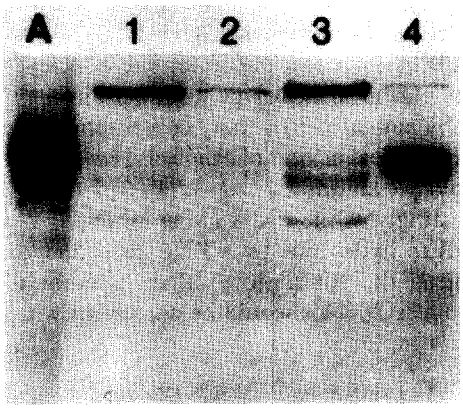

Fig. 2. Western blot analysis of PKC-ε in vector control fibroblasts and in fibroblasts overexpressing PKC-ε. Lanes 1,3 and 2,4 are soluble and particulate fractions, respectively, prepared from vector control fibroblasts (lanes 1,2) and from fibroblasts overexpressing PKC-ε 15-fold (lanes 3,4). 'A' represents rat brain standard.

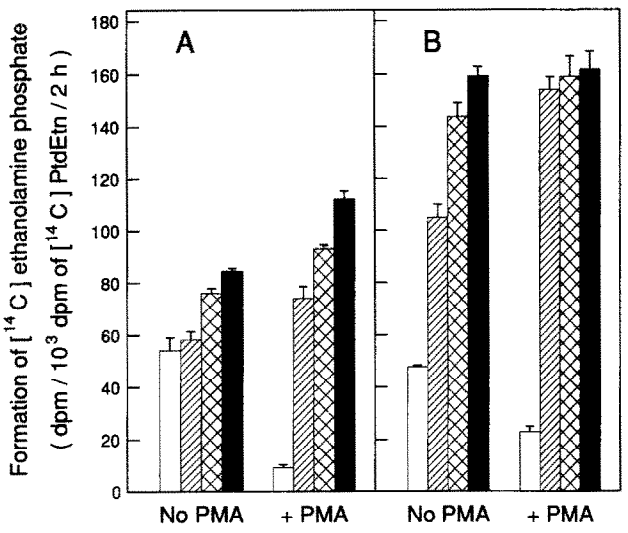

Fig. 3. Potentiation of long-term stimulatory effect of ethanol on PLC-mediated PtdEtn hydrolysis by overexpressed PKC-ε. Vector control NIH 3T3 fibroblasts (A) and fibroblasts overexpressing PKC-ε ~ 15-fold (B) were labeled with [^{14}C]ethanolamine for 48 h, followed by treatments of suspended fibroblasts for 2 h with 100 nM PMA as indicated, in the absence (□) or presence of 37.5 mM (▨), 75 mM (▩), and 150 mM ethanol (■). The cellular content of [^{14}C]PtdEtn in these cells was in the range of 906,000–939,000 dpm/10^6 cells. [^{14}C]EtnP was separated from other hydrolytic products as indicated in section 2. Data are the mean ± S.E.M. of three incubations. Similar results were obtained in two other experiments.

brane fraction (Fig. 1, lane 4). In five experiments examined, membranes from PMA plus 150 mM ethanol-treated fibroblasts (Fig. 1, lane 6) contained 35 ± 13% more PKC-ε than membranes from cells treated with PMA. Ethanol alone increased the cytoplasmic content of PKC-ε (Fig. 1, lane 7) at the expense of the membrane-bound form (Fig. 1, lane 8). Ethanol and PMA had similar synergistic effects on the membrane content of PKC-ε after treatments for 30 min (data not shown). While these effects of ethanol and PMA established an important precondition for the regulation of PLC activity by PKC-ε, these data did not serve as proof that such regulation actually occurs.

To further examine the possible regulatory role of PKC-ε, next we used vector control fibroblasts (NIH 3T3/PKC-ε; Fig. 2, lanes 1,2) and fibroblasts overexpressing PKC-ε (about 15-fold) in which PKC-ε is predominantly located in membranes (NIH 3T3/PKC-ε_{15}; Fig. 2, lanes 3,4). In [^{14}C]ethanolamine-labeled vector control fibroblasts, 33.5 mM ethanol alone had no effect on PtdEtn hydrolysis, while 75 and 150 mM concentrations of ethanol stimulated PLC-mediated formation of [^{14}C]EtnP from [^{14}C]PtdEtn 1.4- and 1.55-fold, respectively (Fig. 3A). Although in vector control cells, PMA (100 nM) alone inhibited PLC activity by 83%, it significantly enhanced the stimulatory effects of ethanol at each concentration employed (Fig. 3A). Overexpression of PKC-ε failed to enhance the 'basal' level of [^{14}C]EtnP formation, and it actually decreased the inhibitory effect of PMA to 50% (Fig. 3B). However, overexpressed PKC-ε greatly enhanced the ability of ethanol to stimulate PLC activity. Thus, in the absence of PMA, 37.5, 75 and 150 mM concentrations of ethanol increased [^{14}C]EtnP formation 2.2–3.0- and 3.35-fold, respectively (Fig. 3B). Importantly, the stimulatory effect of 150 mM (maximally effective concentration) ethanol was not affected by PMA, while the effects of lower concentrations of ethanol were enhanced by PMA to approximately the same level which was achieved by 150 mM ethanol alone (Fig. 3B).

In vector control fibroblasts, ethanol alone or in combination with PMA had no effect on PLC activity after 15 min incubation (Fig. 4). In contrast, in PKC-ε overexpressors 150 mM ethanol alone stimulated PLC-mediated PtdEtn hydrolysis about 2.3-fold; this effect of ethanol was not significantly enhanced by PMA (Fig. 4).

Measurable increase in ethanol effect (in the absence of PMA) on PtdEtn hydrolysis required extensive overexpression of PKC-ε. In this work we also examined the effect of ethanol in two other fibroblast lines overexpressing PKC-ε about 3-fold and 10-fold. In the former line we have not observed significant increase in the stimulatory effect of ethanol, while 10-fold overexpression of PKC-ε approximately doubled the effects of 75 and 150 mM concentrations of ethanol on PtdEtn hydrolysis (data not shown).

In conclusion, we showed here that overexpression of PKC-ε in fibroblasts sensitizes the PtdEtn-hydrolyzing PLC system to the stimulatory effect of ethanol. In PKC-ε overexpressors, stimulation of PLC by a maxi-

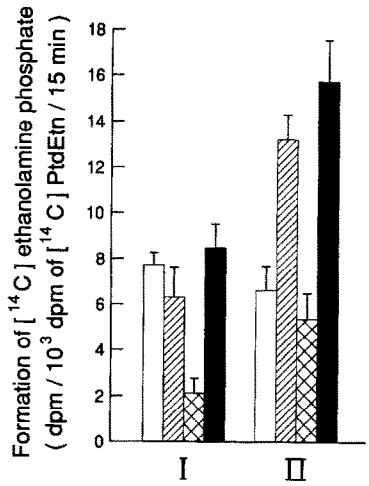

Fig. 4. Potentiation of short-term stimulatory effect of ethanol on PLC-mediated PtdEtn hydrolysis by overexpressed PKC-ε. Vector control NIH 3T3 fibroblasts (I) and fibroblasts overexpressing PKC-ε ~ 15-fold (II) were labeled with [^{14}C]ethanolamine for 48 h, followed by incubation of suspended fibroblasts for 15 min in the absence (□) or presence of 150 mM ethanol (▨), 100 nM PMA (▩), or ethanol plus PMA (■). Data are the mean ± S.E.M. of three incubations. Similar results were obtained in two other experiments.

mally effective concentration of ethanol did not require co-addition of a PKC activator. Evidently, overexpressed PKC-ε alone is not able to regulate PLC activity. Most recently, ethanol has been reported to inhibit calcium-dependent PKC activity [9]. We also showed that PKC-α, the only calcium-dependent PKC-isozyme present in these fibroblasts, inhibits PLC activity [2]. Thus, it is possible that ethanol indirectly promotes an interaction between the PKC-ε and PLC systems by inhibiting the activity of inhibitory PKC-α. Whether this is the case or not, it is clear that the relative amounts of PKC-ε and PKC-α determine organ sensitivity to the stimulatory effect of ethanol on PLC activity.

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