Modulation of mouse estrogen receptor transcription activity by protein kinase C δ

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ABSTRACT

The effect of protein kinase C (PKC) δ on the transcriptional activity of the mouse estrogen receptor was investigated. The receptor was expressed transiently in Cos-1 and NIH3T3 cells in the presence of wild-type, dominant negative or constitutively active forms of PKC δ . Transfection experiments demonstrated that PKC δ stimulated both unliganded and liganded estrogen receptor transcriptional activity. This stimulatory effect was not observed using PKC α or PKC ϵ . 4-Hydroxytamoxifen and the pure anti-estrogen ICI 164,384 reduced receptor transcriptional activity in the presence of PKC δ . The stimulatory effect of PKC δ on estrogen receptor transcriptional activity was mediated by the N-terminal activation

function 1 (AF-1) domain. The reduced stimulatory effect of PKC δ on transcriptional activity of the phosphorylation defective mutant of estrogen receptor suggests that phosphorylation of serine 122 in the AF-1 region may mediate the modulatory effect of PKC δ . Wild-type PKC δ caused a twofold increase in estrogen receptor phosphorylation, while a dominant negative mutant of PKC δ reduced the receptor phosphorylation to five percent of that caused by wild-type PKC δ . Our results suggest that PKC δ participates in the signaling pathways that lead to estrogen receptor phosphorylation and its effect on estrogen receptor transcriptional activation is both cell type and promoter specific. *Journal of Molecular Endocrinology* (1998) **20**, 245–259

INTRODUCTION

The estrogen receptor is a member of a superfamily of nuclear hormone receptors that function as ligand-dependent transcription factors (Green & Chambon 1988, Gronemeyer 1991, Parker 1993). As with other members of this superfamily, it consists of three structural domains: a variable N-terminal domain, a highly conserved DNA binding domain and a moderately conserved C-terminal ligand binding domain (Evans 1988, Parker 1993, Beato *et al.* 1995). Estrogen receptors are located predominantly in the cell nucleus but undergo a process of nucleo-cytoplasmic shuttling (Guiochon-Mantel *et al.* 1991, Dauvois *et al.* 1992). In the absence of hormone the estrogen receptor exists as an inactive oligomeric complex with heat-shock protein hsp90 (Pratt 1993, Smith & Toft 1993). As a result of hormone binding the complex dissociates and the receptor either binds directly to the DNA as a homodimer (Kumar & Chambon

1988, Fawell *et al.* 1990) or after interaction with other transcription factors by protein–protein interaction.

The estrogen receptor stimulates transcription by means of two activation functions, AF-1 and AF-2. AF-1 is located in the N-terminal domain that has constitutive transcriptional activity, while AF-2 requires estrogen binding for its activity and is located in the C-terminal domain (Kumar *et al.* 1987, Webster *et al.* 1988, Lees *et al.* 1989, Tora *et al.* 1989). The relative activities of these activation functions are dependent on both the responsive promoter and cell type. They can function independently or cooperate with each other, but their mechanism of action is unknown (Webster *et al.* 1988, Tora *et al.* 1989, Danielian *et al.* 1992).

There is evidence for cross-coupling between intracellular steroid hormone receptors and membrane bound signaling pathways (Power *et al.* 1991, Ignar-Trowbridge *et al.* 1992, Aronica & Katzenellenbogen 1993, Aronica *et al.* 1994,

Nordeen *et al.* 1994, Patrone *et al.* 1996). The neurotransmitter, dopamine, acting through the D1 receptor was found to stimulate the transcriptional activity of several steroid hormone receptors in the absence of hormone (Power *et al.* 1991, Smith *et al.* 1993). Epidermal growth factor (EGF) has been shown to stimulate transcriptional activity of the estrogen receptor, and phosphorylation of serine 118 in the N-terminal activation domain AF-1 was found to be essential for this effect (Ignar-Trowbridge *et al.* 1993, 1996, Kato *et al.* 1995, Bunone *et al.* 1996). Insulin-like growth factor-I (IGF-I) has also been reported to stimulate the transcriptional activity of the estrogen receptor, and its effect was dependent on the presence of estradiol (Aronica & Katzenellenbogen 1993). When protein kinase A (PKA) was stimulated by treating cells with 8-Br-cAMP, the stimulatory effect on the estrogen receptor transcriptional activity was dependent on the presence of estradiol (Cho & Katzenellenbogen 1993).

The estrogen receptor is a phosphoprotein (Migliaccio *et al.* 1986, Auricchio *et al.* 1987, Lahooti *et al.* 1990a, Washburn *et al.* 1991, Ali *et al.* 1993, Lahooti *et al.* 1994, Le Goff *et al.* 1994, Arnold *et al.* 1995). Phosphorylation has been implicated in a number of different functions including receptor processing and shuttling (De Franco *et al.* 1991, Orti *et al.* 1992, Kuiper & Brinkman 1994), DNA binding (Denton *et al.* 1992) and transcriptional activation (Denner *et al.* 1990, Glineur *et al.* 1990, Hsieh *et al.* 1991, Ali *et al.* 1993, Kato *et al.* 1995, Lahooti *et al.* 1995, Bunone *et al.* 1996). Phosphorylation may be the result of other signaling pathways such as those mediated by PKA (Chalbos *et al.* 1993, Fujimoto & Katzenellenbogen 1994) or protein kinase C (PKC) (Hsieh *et al.* 1991), which regulates many cellular functions, and is also the major cellular mediator of 12-*O*-tetradecanylphorbol-13-acetate (TPA) action (Parker *et al.* 1989, Ohno *et al.* 1991, Nishizuka 1992, Dekker & Parker 1994). TPA mimics the action of diacylglycerol by activating PKC *in vitro*. TPA, by stimulating PKC, is also known as a modulator of gene expression through *cis*-acting DNA elements such as the TPA response element (TRE) (Angel *et al.* 1987, Baeuerle & Baltimore 1988). Activating protein (AP-1) is a major TRE binding transcription factor. There are 11 isoforms of PKC which fall into three subgroups (Dekker & Parker 1994) the nomenclature of which is as follows: the conventional (cPKC) or calcium-dependent isoforms have been designated α , β I, β II and γ . Novel (nPKC) or calcium-independent PKC isoforms (Ohno *et al.* 1988, Mizuno *et al.* 1991) are designated δ , ϵ , η , θ and μ . The third group (ζ and

λ) is called atypical (aPKC) due to the lack of phorbol ester/diacylglycerol binding domain and is thought to be activated by particular inositol phospholipids. Tissue distribution and levels of expression are different among the isozymes. These differences suggest that each PKC isozyme plays a distinct role in living cells.

It has been suggested that estrogen may participate, at a post-transcriptional level, in the induction of PKC δ (Cutler *et al.* 1994) in rat corpora lutea at the end of pregnancy. In view of this effect of estrogen on the level of PKC δ , the effect of PKC δ on the transcription activity of estrogen receptor was examined in transiently transfected Cos-1 and NIH3T3 cells in the presence of wild-type, dominant negative or constitutively active forms of PKC δ .

MATERIALS AND METHODS

Expression vectors and reporter gene

The expression vector for the wild-type mouse estrogen receptor mER (White *et al.* 1987) and the mutant receptors mER 1-339 (Lees *et al.* 1989), mER 182-599 (Lahooti *et al.* 1994), mER G-525R (Fawell *et al.* 1990), mER C-241A/C-244A (Lahooti *et al.* 1994), and mER S-122A (Lahooti *et al.* 1995) were subcloned into the EcoRI site of the mammalian expression vector pMT-2 Ω (Kaufman *et al.* 1989). pERE-BL-CAT containing an estrogen response element (ERE) derived from the vitellogenin A2 promoter upstream of the promoter sequences of the *Herpes simplex* viral thymidine kinase gene linked to the reporter gene chloramphenicol acetyltransferase (CAT) was kindly provided by Professor M G Parker (Imperial Cancer Research Fund, London, UK). The reporter plasmid p(ERE)₂-TATA-CAT containing two consensus EREs 38 basepairs from the TATA box of *Xenopus* vitellogenin B1 gene promoter (−42 to 14) was kindly provided by Professor D Shapiro (University of Illinois, Urbana, IL, USA). The expression vectors for wild-type, dominant negative and constitutively active mouse PKC δ (Hirai *et al.* 1994) were kindly provided by Dr S-i Hirai (Yokohama City University, Japan). Expression vectors for PKC α and PKC ϵ were kindly provided by Dr P J Parker (Imperial Cancer Research Fund, London, UK).

Cell culture and transient transfection experiments

Cos-1 and NIH3T3 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 5% (v/v) fetal calf serum (Biowhittaker, Wokingham, UK). Cells were plated out at 2×10^5 cells/28 cm² dish in phenol red-free DMEM, dextran-charcoal treated 5% fetal calf serum and transfected using a liposome-mediated transfection reagent DOTAP (Boehringer, Mannheim, Germany).

Transfected DNA included 2.5 μ g of the reporter plasmids pERE-BL-CAT or p(ERE)₂-TATA-CAT, 0.5 μ g of an internal control plasmid (pGL2 luciferase; Pharmacia, Uppsala, Sweden), 0.25 μ g of the appropriate receptor expression vector as indicated in the Results, 0.2 μ g of the expression vector for PKC δ wild-type, dominant negative or constitutive active and pMT-2 Ω as carrier DNA, to a total of 3.5 μ g/dish. Following transfection, cells were maintained in DMEM with serum-free supplements (Biowhittaker) and in the presence or absence of 10^{-8} M estradiol, 10^{-6} M 4-hydroxytamoxifen, 10^{-7} M ICI 164,384 or 50 nM TPA. Generally after 24 h or 48 h (as indicated in the figure legends) the cells were harvested in reporter lysis buffer (Promega, Madison, WI, USA) and extracts assayed for luciferase activity by using a luciferase assay system (Promega). Aliquots containing equal amounts of protein were used to normalize for variation in cell numbers and the levels of CAT protein in these aliquots were determined by a CAT-ELISA method (Boehringer). Luciferase activity was used to correct for the differences in transfection efficiency in all experiments. Transcription activation is expressed as fold induction over the level of CAT activity in cells expressing receptor in the absence of hormone.

Western blotting of estrogen receptor

Equal amounts of proteins were separated by electrophoresis on 10% (w/v) SDS-polyacrylamide gel and transferred to nitrocellulose in a buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, 20% v/v methanol. After blocking with PBS containing 1% non-fat dried milk and 0.1% Tween-20 (pH 7.5), the receptor was detected with monoclonal antibody H222 using peroxidase-conjugated rabbit anti-rat immunoglobulin G (Dakopatts, Copenhagen, Denmark) as previously described (Lahooti *et al.* 1994). The blots were washed in PBS containing 0.1% Tween-20 (pH 7.5) and developed in enhanced chemiluminescence reagent (Amersham Life Science, Amersham, Bucks, UK) according to the manufacturer's instructions. Autoradiographs were scanned on an LKB Ultrascan XL Laser densitometer (LKB, Rockville, MD, USA). Standard curves were prepared to ensure that the signals were within the linear range of detection.

DNA binding assay

The DNA binding activity of receptor was assayed in a gel shift assay using equal amounts of receptor as determined by Western blotting and a ³²P-labeled double-stranded oligonucleotide probe corresponding to a 32-basepair fragment of the vitellogenin A2 gene containing a consensus ERE sequence (5'-CTAGAAAGTCAGGTCACAGTGACCTGATCAAT-3') as previously described (Fawell *et al.* 1990) with the exception that samples were incubated for 15 min at room temperature. Specific estrogen receptor antiserum MP16 (Fawell *et al.* 1990) was added to the binding reaction as indicated.

Radiolabeling and immunoprecipitation of estrogen receptor

Cos-1 cells were harvested at 70% confluency and, after suspension in PBS, 20×10^6 cells per ml were transfected by electroporation with (5–15 μ g) mER 1-599 in the absence or presence of 1 μ g wild-type (wt PKC δ), dominant negative (dn⁻) and constitutively active (dn⁺) PKC δ , together with 1 μ g pGL2 luciferase (Pharmacia) as an internal control for transfection efficiency. Electroporation was performed at 450 V and 250 μ Farad using a Bio-Rad Gene pulser as previously described (Dauvois *et al.* 1992). Electroporated cells were grown for 24 h in DMEM without phenol red supplemented with 5% (v/v) dextran-charcoal treated (DCC) fetal calf serum (Sigma, St Louis, MO, USA). The cell suspensions were divided between two 9 cm dishes and grown at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. After 24 h, cells were rinsed twice with the serum-free supplemented DMEM and maintained in the same medium for another 24 h. Approximately 36 h after the start of transfection, cells were washed in culture medium without phenol red, phosphate, and methionine. Cells were radiolabeled for 3 h with 500 μ Ci/ml [³²P]orthophosphate or 200 μ Ci/ml [³⁵S]methionine as previously described (Lahooti *et al.* 1994, 1995). All radioisotopes were purchased from Amersham, UK. Cells were stimulated by addition of 50 nM TPA for 3 h prior to harvesting. Cells were collected and whole cell extracts prepared in high salt lysis buffer (400 mM KCl, 50 mM Hepes (pH 7.6), 1.5 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.5 mg/ml bacitracin, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin, 100 μ M sodium vanadate, 20 mM sodium fluoride, 20 mM β -glycerol phosphate and 10 mM sodium molybdate (Lahooti *et al.* 1994). The protein concentration of each extract was determined

by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Radiolabeled estrogen receptors were quantitated after immunoprecipitation with the human estrogen receptor antibody H222 as described by Lahooti *et al.* (1994) with the exception that each labeled band from dried SDS/10% polyacrylamide gels was cut and then solubilized by incubation at 65 °C for 3 h in Soluene (Packard Instruments, Meriden, CT, USA). Samples were counted in Opti Fluor scintillation cocktail using a β -scintillation counter. The amount of receptor in individual samples was initially assessed from [35 S]methionine incorporation and then equal amounts of radiolabeled receptor were analyzed by Western blotting. 32 P incorporation was detected by autoradiography and quantitated by counting 32 P-labeled bands in a β -scintillation counter.

RESULTS

PKC δ stimulates estrogen receptor transcriptional activation

The effect of PKC δ on the transcriptional activity of the mouse estrogen receptor (mER) was analyzed in transiently transfected Cos-1 cells using the reporter plasmid pERE-BL-CAT (Fig. 1A and B). Estrogen receptor stimulated transcription with no added hormone and this was further increased approximately 2.5-fold in the presence of 10 nM estradiol. The anti-estrogen, 4-hydroxytamoxifen, stimulated receptor transcription activity to approximately 70% of hormone-induced activation, while the pure anti-estrogen ICI 164,384 had no stimulatory effect above basal transcription activity (Fig. 1A, mER).

When cells transfected with mER were treated with TPA, transcription was stimulated by estrogen receptor 3-fold in the absence of hormone and further increased another 1.5-fold in the presence of estradiol. Both anti-estrogens reduced the effect of TPA on receptor transcriptional activity in the absence of estradiol (Fig. 1A, mER+TPA). The stimulatory effect of TPA on ER transcription activity suggests that PKC δ increases the transcription activity of mER.

We next investigated whether the PKC δ isoform mediates this effect. Stimulation of cells transfected with both mER and wt PKC δ exhibited an increase in receptor-mediated transactivation of reporter gene of approximately 2.5-fold in the absence of hormone and a further 3-fold increase in the presence of estradiol (Fig. 1A, mER+wt PKC δ +TPA). To show that activation of PKC δ alone can stimulate estrogen receptor transcriptional activity, a constitutively active PKC δ mutant (dn $^{+}$)

was used. Estrogen receptor stimulated transcription 4-fold with no added hormone and a further 4-fold increase occurred in the presence of estradiol when the receptor was expressed with the constitutively active mutant (Fig. 1A, mER+dn $^{+}$ PKC δ). Both anti-estrogens reduced the stimulatory effect of constitutively active PKC δ mutant on receptor transcriptional activity in the absence of estradiol. The strong enhancement of reporter gene expression by constitutively active PKC δ confirms the ability of PKC δ to stimulate receptor transcriptional activity.

The activation of transcription by the wild-type or the constitutively active form of PKC δ in the absence of receptor was negligible (Fig. 1A, wt PKC δ +TPA). Estrogen receptor stimulated transcription approximately 2- to 4-fold, without TPA addition, in the presence of wild-type PKC δ (data not shown). This might reflect partial activation of exogenous PKC δ , which took place before the cells were transferred to complete serum-free medium.

The requirement of PKC δ for the increase in receptor transcriptional activity was assessed by using a dominant negative PKC δ mutant (dn $^{-}$). Estrogen receptor stimulated transcription in the absence of hormone and this was further increased approximately 4-fold in the presence of 10 nM estradiol (Fig. 1B, mER). Fig. 1B shows that in the presence of wild-type or dn $^{+}$ PKC δ exposure to 10 nM estradiol caused approximately 6- and 15-fold increases respectively in estrogen receptor-mediated transcription. Cotransfection with dn $^{-}$ PKC δ resulted in a dose-dependent inhibition of this stimulatory effect (Fig. 1B, mER+wt PKC δ +dn $^{-}$ and mER+dn $^{+}$ PKC δ +dn $^{-}$). The data of Fig. 1A and B suggest that PKC δ stimulated transcription activation of both unliganded and liganded receptor.

The above results could be interpreted to have been caused by increased estrogen receptor expression and by an increased capacity of the receptor for binding to ERE. These were excluded as follows: the levels of receptor protein (Fig. 2) were measured by Western blotting (Fig. 2A) using monoclonal antibody H222 raised against the human estrogen receptor (Greene *et al.* 1984). It was found that although there were slight variations in the amount of receptor protein, there was no correlation between transcription activity and estrogen receptor concentration. Equivalent amounts of receptor protein, as determined by Western blotting, were incubated with a DNA probe consisting of a 32 P-labeled oligonucleotide containing a consensus ERE. Receptor DNA binding activity was indicated by the retardation of the 32 P-labeled ERE on a polyacrylamide gel (Fig. 2B). The reduction in

FIGURE 1. (A) The effect of wild-type (wt PKC δ) and constitutively active PKC δ (dn⁺) on the transcription activity of mouse estrogen receptor (mER). Transcription activity of mER was analyzed in transiently transfected Cos-1 cells using reporter plasmid pERE-BL-CAT. Cells were maintained in serum-free supplemented medium in the absence of added ligand (no hormone), or in the presence of 10^{-8} M estradiol, 10^{-6} M 4-hydroxytamoxifen, or 10^{-7} M ICI 164,384, or 50 nM TPA for 24 h. Aliquots containing equal amounts of protein from each sample cell extract were incubated with CAT antibody for 1 h at 37 °C. Transcription activation is expressed as fold induction over the level of CAT activity in cells expressing receptor in the absence of hormone. The error bars represent standard error of the mean (S.E.M.) determined from three experiments, each carried out in duplicate. The expression vector alone is shown as Ω . (B) The effect of increasing amounts of dominant negative PKC δ (dn⁻) on the transcription activity of mER in the presence of a constant amount (0.2 μ g) of wild-type and constitutively active PKC δ . Control represents activity in the absence of dn⁻. The ladders in Fig. 1B represent the increasing amounts of dominant negative PKC δ . The error bars represent the range for the mean of two experiments, each carried out in duplicate.

FIGURE 2. Receptor protein was quantitated by (A) Western blotting and (B) gel shift assay. Wild-type estrogen receptor was expressed transiently in Cos-1 cells in the absence of hormone (lane 2) or in the presence of 50 nM TPA (lane 3), wild-type PKC δ treated with 50 nM TPA (lane 4), dominant negative mutant treated with 50 nM TPA (lane 5), constitutively active mutant in the absence of TPA (lane 6), a combination of wild-type plus dominant negative treated with 50 nM TPA (lane 7) or a combination of constitutively active plus dominant negative (lane 8). In the gel shift assays presented in panel (B), the above samples (lanes 3–9) were performed in the absence of estradiol. The receptor DNA binding activity was indicated by the retardation of the 32 P-labeled ERE on a polyacrylamide gel. The estrogen receptor-DNA complexes were identified by using a specific estrogen receptor antiserum MP16. Ω indicates a control vector. The positions of molecular weight markers are given in kilodaltons on the right hand side of panel (A).

DNA binding activity of receptor expressed with wild-type or constitutively active PKC δ suggests that the stimulatory effect of PKC δ on receptor transcriptional activity is not mediated by an increase in DNA binding activity (Fig. 2B lanes 4, 6, 7 and 8).

PKC δ -mediated transcription activation of estrogen receptor requires N-terminal AF-1

The effects of PKC δ on the transcription activity of a number of mutant estrogen receptors (Fig. 3A) were analyzed in transiently transfected Cos-1 cells using reporter pERE-BL-CAT. The ability of altered mutant receptors to stimulate transcription was used to evaluate which of the receptor activation functions participate in the PKC δ stimulatory effect (Fig. 3B). The wild-type estrogen

receptor (mER) stimulated transcription 5-fold in the presence of 10 nM estradiol and this effect was reduced to 2.5-fold and a negligible level in the presence of 4-hydroxytamoxifen and ICI 164,384 respectively. Constitutively active PKC δ (mER+dn $^{+}$) stimulated transcriptional activity of the unliganded (3-fold) and liganded (19-fold) wild-type estrogen receptor, and this effect was considerably reduced in the presence of anti-estrogens.

When the deletion mutant mER 1-339 (Fig. 3A and B) was tested, the hormone-independent transcriptional activity of AF-1 alone was 2- to 3-fold that of wt mER. The 4-hydroxytamoxifen inhibited AF-2 transcription activation for both wt and 1-339 receptors was comparable being about 3-fold greater than hormone-free wt mER. With AF-1 alone transcriptional activity increased 6- to 14-fold in the presence of dn $^{+}$ (mER 1-339+dn $^{+}$) regardless of the presence or absence of estrogen and anti-estrogens.

To determine the effect of PKC δ upon the activity of AF-2 in the absence of AF-1, the deletion mutant mER 182-599 (Fig. 3A and B) was also tested. This construct lacks the entire N-terminal domain but retains estrogen and DNA binding activities. Both anti-estrogens reduced transcriptional activity of AF-2 to undetectable levels with this deletion mutant (Fig. 3B mER 182-595). Constitutively active PKC δ stimulated basal but not estradiol-mediated transcriptional activity of N-terminally truncated receptor mutants (Fig. 3B, mER 182-599+dn $^{+}$). Thus, a region between amino acids 1-182 is necessary for mediating the stimulatory effect of PKC δ on estradiol-mediated transcriptional activity.

Next, the importance of ligand in the PKC δ effect on estrogen receptor transcriptional activity was examined by using the mutant receptor mER G-525R (Fig. 3A and B). This mutant fails to bind estradiol at a concentration of 50 nM, but it retains its sensitivity to 4-hydroxytamoxifen and is able to bind DNA and stimulate transcription by means of AF-1 (Danielian *et al.* 1993). The ability of mER G-525R alone to show a transcriptional response to 4-hydroxytamoxifen increased nearly 10-fold in the presence of constitutively active PKC δ (Fig. 3B, mER G-525R+dn $^{+}$). Constitutively active PKC δ could not stimulate the transcriptional activity of this mutant in the presence of 10 nM estradiol due to its defective estrogen binding (Fig. 3B, mER G-525R+dn $^{+}$). Thus, this experiment suggests that the stimulatory effect of PKC δ is, at least in part, on the transcription activation of unliganded wild-type receptor, and this effect is enhanced upon binding estradiol or 4-hydroxytamoxifen. When the

FIGURE 3. (A) Schematic organization of the full-length mouse estrogen receptor (mER 1-599) deletion and point mutants investigated in the study. The receptor is divided into three domains: the N-terminal activation domain AF-1, the DNA binding domain (DBD), and the hormone binding domain that contains another activation domain, AF-2, as well as nuclear localization (NL) signals in the hinge region. Positions of phosphorylated serines are indicated by a P within a circle in the domain structure of the receptor. (B) The effect of constitutively active PKC δ (dn⁺) on the transcriptional activity of wild-type (mER) and mutant estrogen receptors was analyzed in transiently transfected Cos-1 cells using reporter pERE-BL-CAT. Cells were maintained in serum-free supplemented medium in the absence of added ligand (no hormone), or in the presence of 10^{-8} M estradiol, 10^{-6} M 4-hydroxytamoxifen, or 10^{-7} M ICI 164,384 for 24 h. Aliquots containing equal amounts of protein from each cell extract were incubated with CAT antibody overnight at 4 °C. Transcriptional activation is expressed as fold induction over the level of CAT activity in the absence of hormone, in cells expressing receptor. Error bars represent the range for the mean of two experiments, each carried out in duplicate. The expression vector alone is shown as Ω .

FIGURE 4. The effect of PKC δ (dn⁺) as well as PKC α or PKC ϵ on the transcription activity of wild-type (mER) receptors was analyzed in transiently transfected NIH3T3 (A and B) or Cos-1 (C and D) cells using reporter genes pERE-BL-CAT (A and C), or p(ERE)₂-TATA-CAT (B and D). Cells were maintained in serum-free supplemented medium either in the absence of added ligand (solid bars) or in the presence of 10^{-8} M estradiol (open bars) for 48 h. TPA (50 nM) was added during the last 6 h of 48-h post-transfection or the entire 48-h post-transfection as indicated. Aliquots containing equal amounts of protein from each cell extract were incubated with CAT antibody overnight at 4 °C. Transcriptional activation is expressed as fold induction over the level of CAT activity in the absence of hormone, in cells expressing receptor. Error bars for (A), (B) and (D) represent the range for the mean of two experiments, each carried out in duplicate. Error bars for (C) represent standard error of the mean (S.E.M.) for three experiments each carried out in duplicate. The expression vector alone is shown as Ω .

DNA binding defective receptor mutant C-241A/C-244A (Fig. 3A) was used, the effect of PKC δ on transcriptional activity of the estrogen receptor was negligible (data not shown).

Serine 122 in the AF-1 region of the estrogen receptor is phosphorylated through the mitogen activated protein (MAP) kinase pathway. Phosphorylation of serine 122 may also modulate the effect of PKC δ on estrogen receptor transcription activity. The effect of PKC δ (dn⁺) on the transcription activity of phosphorylation defective mouse estrogen receptor mutant (Fig. 3A, mER S-122A) was analyzed in transiently transfected

Cos-1 (Fig. 3B) and NIH3T3 (Fig. 4A) cells. PKC δ in Cos-1 cells in the presence of 10 nM estradiol stimulated transcription 1.5-fold (compare mER S-122A with mER S-122A+dn⁺). The PKC δ effect on transcription activity of mER S-122A+dn⁺ in the presence of 10 nM estradiol was negligible in NIH3T3 cells in contrast to the 14-fold potentiation observed with wild-type mER+dn⁺. These experiments suggest that the phosphorylation of serine 122 in the AF-1 region may be important for mediating the stimulatory effect of PKC δ on estrogen receptor transcription activity and it is possibly cell type specific.

PKC α and PKC ε do not stimulate estrogen receptor transcription activation

The effects of two wild-type calcium-dependent PKC isoforms PKC α and PKC ε , on the transcription activity of the wild-type mouse (Fig. 4A-D, mER) estrogen receptor were analyzed in transiently transfected NIH3T3 (Fig. 4A and B) and Cos-1 (Fig. 4C and D) cells using the reporter plasmids pERE-BL-CAT (Fig. 4A and C) or p(ERE)₂-TATA-CAT (Fig. 4B and D) containing complex or simple promoters respectively. Wild-type estrogen receptor stimulated transcription approximately 3- to 5-fold in the presence of 10 nM estradiol using either pERE- BL-CAT (Fig. 4A and C) or p(ERE)₂-TATA-CAT (Fig. 4B and D) reporters. PKC δ (dn⁺) stimulated basal (Fig. 4A,C,D, mER+dn⁺) and estradiol dependent (Fig. 4A-D, mER+dn⁺) receptor transcription activity. In the presence of dn⁺ in NIH3T3 cells, using the simple reporter p(ERE)₂-TATA-CAT (Fig. 4B, mER+dn⁺), the basal transcription activity of mER was reduced to 50% of control (Fig. 4B, mER in the absence of hormone). In contrast to the stimulatory effect of PKC δ on receptor transcription activation, PKC α and PKC ε reduced both the basal and estradiol-stimulated transcription using either of the reporters in the two cell lines tested (Fig. 4A-D, mER+wt PKC α $\pm$ TPA or mER+wt PKC ε $\pm$ TPA). Addition of TPA for the last 6 h of the 48-h post-transfection considerably reduced the basal and estradiol-induced transcription activation of both reporters (Fig. 4A-D, mER+TPA 6 h) in NIH3T3 and Cos-1 cells. Addition of TPA for the entire 48-h post-transfection period had a positive stimulatory effect on basal and estradiol-induced transcription activation of both reporter genes in the two cell lines tested. The receptor proteins expressed in the transient study with NIH3T3 cells were difficult to detect by gel shift analysis and Western blotting. Therefore, the expression of wild-type receptor protein was studied by immunofluorescence, using the monoclonal antibody H222 raised against the human estrogen receptor (Greene *et al.* 1984). It was found that the transfected cells contained wild-type receptor proteins in similar amounts, and were located in the cell nucleus (data not shown). These experiments suggest that PKC δ stimulates while PKC α and PKC ε have an inhibitory effect on the estrogen receptor transcriptional activity.

Phosphorylation of estrogen receptor by protein kinase C δ

The effect of PKC δ on the phosphorylation of the mouse estrogen receptor (Fig. 5B) was analyzed in

FIGURE 5. Phosphorylation of mouse estrogen receptor. Mouse estrogen receptor (mER) was expressed in Cos-1 cells in the absence of hormone (lane 2) or in the presence of 50 nM TPA (lane 3), a combination of wild-type plus dominant negative (lane 4; wt+dn⁻ PKC δ +TPA), dominant negative mutant (lane 5; dn⁻ PKC δ +TPA), constitutively active mutant (lane 6; dn⁺ PKC δ) or wild-type (lane 7; wt PKC δ +TPA) PKC δ . (A) The amounts of receptor protein were quantitated by Western blotting. (B) [³²P]Orthophosphate incorporation was detected by autoradiography. Ω indicates a control vector (lane 1). The positions of molecular weight markers are given in kilodaltons on the right.

transiently transfected Cos-1 cells in the presence of wild-type (wt), dominant negative (dn⁻) and constitutively active (dn⁺) PKC δ . The amount of receptor in individual samples was assessed from [³⁵S]methionine incorporation, and then equal amounts of radiolabeled receptors were analyzed by Western blotting. We found that the relative amounts of receptor determined by [³⁵S]methionine incorporation (data not shown) and Western blotting were similar (Lahooti *et al.* 1994). Radiolabeled whole cell extracts, immunoprecipitated with normal rat serum, gave no equivalent band (data not shown). Receptor phosphorylation was slightly increased in the presence of TPA (1.3-fold, lane 3), constitutively active PKC δ (1.5-fold, lane 6) and wild-type PKC δ (2-fold, lane 7) (Fig. 5B), when equal amounts of estrogen receptor, based upon quantitation by Western blotting, were used (Fig. 5A). The dominant negative mutant of PKC δ (Fig. 5B, dn⁻+TPA, lane 5) reduced receptor phosphorylation to five

percent of that by the wild-type PKC δ . Coexpression of dominant negative with wild-type PKC δ (Fig. 5B, wt+dn⁻ PKC δ +TPA, lane 4) resulted in a slight decrease (0.8-fold) in receptor phosphorylation. The reduced level (0.1-fold) (Fig. 5B, lane 5) of receptor phosphorylation in the presence of dominant negative mutant (Fig. 5B, dn⁻+TPA) was not due to the action of phosphatases, since inhibition of endogenous phosphatases by incubating the cells expressing the dominant negative mutant with okadaic acid, still resulted in reduced receptor phosphorylation (data not shown). Thus, these results suggest that PKC δ participates in estrogen receptor phosphorylation.

DISCUSSION

In the present study the effect of PKC δ on the transcriptional activity of the mouse estrogen receptor was analyzed in transiently transfected Cos-1 and NIH3T3 cells using both complex (pERE-BL-CAT) and simple (p(ERE)₂-TATA-CAT) estrogen responsive reporter genes. The results reported in this study suggest that PKC δ stimulates unliganded and liganded estrogen receptor transcriptional activation and that serine 122 in the N-terminal AF-1 domain may be important for mediating this effect.

A number of approaches were used to confirm that PKC δ stimulates estrogen receptor transcriptional activity. First, three selected isozymes PKC α , PKC ϵ , and PKC δ were expressed in Cos-1 and NIH3T3 cell lines and their effect on the induction of pERE-BL-CAT and p(ERE)₂-TATA-CAT expression by TPA was tested. It was found that PKC δ stimulated, while PKC α and PKC ϵ reduced, estrogen receptor transcriptional activity. Secondly, expression of a constitutively active PKC δ mutant (dn⁺), in the two cell lines, demonstrated that the activation by PKC δ alone was sufficient for stimulating receptor transcriptional activation. Thirdly, a dominant negative mutant of PKC δ (dn⁻) was used to test the requirement of PKC δ for stimulating estrogen receptor-mediated activation of gene expression. The dominant negative PKC δ mutant inhibited in a dose-dependent manner the stimulatory effect of both wild-type and constitutively active PKC δ on estrogen receptor transcription activation (Fig. 1B). It is possible that the inhibitory effect of the dominant negative mutant results from its binding to a common substrate that is normally associated with wild-type PKC δ . Anti-estrogens were used to show that the stimulatory effect of PKC δ on receptor transcription activity is receptor-mediated. Deletion, as

well as point mutation of the estrogen receptor, were used to evaluate the role of receptor domains in the effect of PKC δ on receptor transcriptional activity. The ability of PKC δ to increase the AF-1 activity of the mER G-525R (Danielian *et al.* 1993) mutant that has retained its sensitivity to 4-hydroxytamoxifen, indicates that the effect is on transcription activation of unliganded wild-type estrogen receptor. This effect is enhanced upon binding of either estradiol or 4-hydroxytamoxifen (Fig. 3B, mER G-525R+dn⁺). This opens the possibility that increased agonistic activity of 4-hydroxytamoxifen on AF-1 activity by PKC δ contributes to tamoxifen resistance in some breast cancers.

Previously, a reduction of transcriptional activity was observed when serine 122 was mutated in conjunction with mutations in a putative amphipathic α -helix involved in AF-2 activity (Lahooti *et al.* 1995). It was suggested that a region of AF-1 that encompasses serine 122 appears to interact with AF-2 in the full-length receptor (Lahooti *et al.* 1995). Serine 122 in the mouse receptor is equivalent to serine 118 in the human receptor and phosphorylation at this residue occurs in a region of AF-1 that may be important for mediating the stimulatory effect of PKC δ on receptor transcriptional activation. This effect was demonstrated by the inability of the phosphorylation defective mutant receptor (mER S-122A+dn⁺) to respond to the stimulatory effect of PKC δ , and the reduced level of transcription observed with mutant receptor, compared with that of the wild-type receptor protein (Figs 3B and 4A, mER S-122A+dn⁺). Serine 122 represents a potential target for phosphorylation by extracellular signal-regulated kinases ERK1/ERK2 that are activated by growth factors through the MAP kinase pathway. It has been reported that treatment of Cos-1 cells expressing human estrogen receptor with EGF or IGF-I enhanced the AF-1 activity (Kato *et al.* 1995, Bunone *et al.* 1996) and this effect was dependent on the phosphorylation of serine 118 by the Ras-Raf-mitogen activated protein kinase (MAPK) cascade. However, other Ras-independent MAPK activation pathways (Hill & Treisman 1995) might also increase the activity of the estrogen receptor AF-1 domain. It is possible that the stimulatory effect of PKC δ on transcription activation of receptor is mediated by protein-protein interaction, and that phosphorylation of serine 122 may function as a signal for recognition of the receptor protein by other factors (co-activators), such as receptor-interacting proteins (RIPs) RIP160 and RIP140 (Cavailles *et al.* 1994, Halachmi *et al.* 1994) or steroid receptor coactivator-1 (SRC-1) (Onate *et al.* 1995).

The stimulatory effect of PKC δ on estrogen receptor transcriptional activity could also be a result of changes in conformation of unactivated oligomeric receptor complexes. Previously, we have shown that estradiol causes a rapid increase of hsp90 phosphorylation in MCF-7 cells, and this effect is also modulated by Ca^{2+} -independent PKC activity (Lahooti *et al.* 1990b). It is, therefore, conceivable that phosphorylation of any one of the components of the unactivated receptor (Pratt & Toft 1997) by PKC δ , may result in dissociation of the oligomeric complex, leading to the activation of receptor. This effect should be independent of hormone, but could be potentiated in the presence of hormone. The stimulatory effect of PKC δ on estrogen receptor transcription activity can also be explained by the recent model for the assembly and disassembly of hsp90–receptor complexes (Smith 1993). In the Smith ‘disactivation loop model’, complexes continuously disassemble, but are reassembled through a very efficient and ATP-dependent pathway. Considering this model, PKC δ might act upon the receptor in its disassembled state to affect receptor transcriptional activation, shifting the equilibrium towards the disassembled state by preventing the receptor molecules from reassociating with hsp90. The disassembled receptor molecules that are now in an active state, can bind to the target gene DNA to activate transcription. In view of the ability of estradiol to increase the phosphorylation of hsp90, it is conceivable that the resulting increase in the surface negative charge of hsp90 may prevent the association of active receptor molecules with hsp90, resulting in an increase in receptor transcriptional activity. A recent observation by White *et al.* (1997) suggested that, in the absence of hormone, a conformational change in the estrogen receptor produces an interacting surface that may associate with potential coactivator proteins, resulting in receptor transcriptional activity.

Our results suggest that the effect of PKC δ on estrogen receptor transactivation is both cell type and promoter specific. Using a reporter plasmid with a simple promoter (p(ERE)₂-TATA-CAT), PKC δ stimulated both basal and hormone-induced transcriptional activation of receptor in Cos-1 cells (Fig. 4D), but in NIH3T3 cells PKC δ reduced the basal transactivation of estrogen receptor (Fig. 4B). Promoter specificity in NIH3T3 cells (Fig. 4A and B) was observed with PKC δ stimulating the basal activation of estrogen receptor using the complex reporter gene (Fig. 4A), while the simple promoter resulted in PKC δ inhibiting basal expression (Fig. 4B). In contrast to the stimulatory effect of PKC δ , PKC α and PKC ϵ reduced estrogen receptor transcriptional activity. In preliminary

experiments a similar effect of PKC α and PKC ϵ was observed in MCF-7 cells (data not shown). Addition of TPA for the last 6 h of the 48-h post-transfection period considerably reduced the basal and estradiol-induced gene activation of both reporters in NIH3T3 and Cos-1 cells (Fig. 4A–D). However, addition of TPA during the entire 48-h post-transfection period had a positive regulatory effect, in which the basal and estradiol-induced transcription activity of the receptor returned to control levels in both cell lines and in the presence of both reporters (Fig. 4A–D). The effect of TPA on estrogen receptor gene activation is cell and promoter specific, and probably requires the synthesis or activation of a factor(s) following stimulation (Cho & Katzenellenbogen 1993, Martin *et al.* 1995).

Phosphorylation of receptor protein increased (2-fold) in the presence of wild-type PKC δ +TPA (Fig. 5B) while TPA alone slightly stimulated receptor phosphorylation. Recently, it has been reported that TPA caused phosphorylation of serine 118 of the human estrogen receptor expressed in Cos-1 cells (Le Goff *et al.* 1994, Joel *et al.* 1995). The TPA stimulation of receptor phosphorylation may be related to the activation of MAP kinases. In a recent publication Ueda *et al.* (1996) reported that protein kinase C δ activates the mitogen extracellular signal regulated kinases (MEK-ERK) pathway in a manner that was dependent on Raf but independent of Ras. The 14-3-3 proteins are associated with PKC and Raf-1; Wakui *et al.* (1997) demonstrated a stimulatory effect of 14-3-3eta protein on transcriptional activation by glucocorticoid receptor.

Our results suggest that PKC δ participates in the signaling pathways that lead to receptor phosphorylation. We do not believe that PKC δ is the kinase responsible for phosphorylation of estrogen receptor, based on the different compartmentalization of the two proteins. Its effect on receptor phosphorylation is most likely mediated through the MAP kinase pathway (Kato *et al.* 1995, Bunone *et al.* 1996, Ignar-Trowbridge *et al.* 1996). In this report we have shown that the dominant negative mutant of PKC δ reduces receptor phosphorylation, which indicates interaction with a common substrate or binding protein that may mediate the phosphorylation of receptor by MAPK. PKC δ , as well as PKC α , are able to activate MAPKs, and continuous activation of PKC is essential for cell growth and differentiation (Watanabe *et al.* 1992, Yamaguchi *et al.* 1995).

The reduction in the DNA binding activity of receptor expressed in the presence of PKC δ was not the result of a decreased affinity of the receptor

FIGURE 6. A tentative model for the interaction between estrogen receptor and PKC δ pathway. Growth factors bind to their receptors, resulting in autophosphorylation (P) of tyrosine residues (Y). This is a recognition signal for a number of adaptor proteins to transmit the signal for activation of Ras. Activation is followed by recruitment of Raf-1 kinase to the plasma membrane and its interaction with the 14-3-3 protein. Raf-1 may be activated by another protein belonging to the PKC family. The activated Raf-1 further transmits the signal to the next kinase, MEK (MAPKK), which is also a substrate for PKC δ . Activated MEKs subsequently activate the MAPKs, ERK1 and ERK2. Once activated, MAPK is translocated to the nucleus and phosphorylates a large number of regulatory proteins. Phosphorylated estrogen receptor (circled p) can then activate transcription of target genes either by directly binding to the ERE, or indirectly via interaction with auxiliary proteins. It is also able to stimulate an AP-1 response element, or cooperates with AP-1 proteins to stimulate ERE-dependent transcription, resulting in growth, development and differentiation. PLC, phospholipase C; Grb2, growth factor regulated binding protein 2; Sos, son of sevenless.

for DNA (data not shown). Competition experiments also suggested no influence of PKC δ and its mutants on the binding of estrogen receptor to the ERE (data not shown). It is possible that the

reduced DNA binding activity of the receptor in the presence of PKC δ is a reflection of the amount of the disassembled receptor; however, once the PKC δ activated receptor is bound to the DNA it is able

efficiently to initiate transcription to a much higher degree than in the absence of PKC δ . This acquired efficiency of the receptor transcriptional activity may be achieved by phosphorylation of the receptor.

Although the exact mechanism of the PKC δ activation of estrogen receptor transcriptional activity is currently unknown, possible signal transduction pathways are presented in Fig. 6. Growth factors bind to their receptors, resulting in activation of the MAPKs, ERK1 and ERK2, that phosphorylate a large number of regulatory proteins that directly control several cellular processes including transcription, translation and cytoskeletal rearrangement. The estrogen receptor stimulation of a tyrosine kinase pathway in MCF-7 cells (Migliaccio *et al.* 1996) demonstrates the involvement of multiple signaling pathways in the function of estrogen receptor and possibly other nuclear receptors. It remains to be seen what effect estradiol will have on the PKC δ and MAPK activities, the activation of MAPK pathway by PKC δ , the roles of covalent modification of co-activators and co-repressor binding, and how they modify the ligand effects.

ACKNOWLEDGEMENTS

We are very grateful to Professor M G Parker for providing estrogen receptor expression plasmids and reporter plasmid ERE-BL-CAT, to Professor D Shapiro for reporter plasmid p(ERE)₂-TATA-CAT, to Dr S-i Hirai for PKC δ expression plasmids, and to Dr P J Parker for PKC α and PKC ϵ expression plasmids. We are very grateful to Professor G Greene, Dr A Wakeling and Dr E Lien for providing estrogen receptor antibody H222, and the anti-estrogens ICI 164,384 and 4-hydroxytamoxifen respectively. We thank Professor O L Myking for helpful discussion and Mrs C Cooke for technical assistance. We thank Drs P J Mark and T Ratajczak for critical reading of the manuscript. This research was supported by grants from the Norwegian Cancer Society.

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RECEIVED 26 August 1997