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The Protein Kinase C Activator, Phorbol Ester, Elicits Disparate Functional Responses in Androgen-Sensitive and Androgen-Independent Human Prostatic Cancer Cells

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The protein kinase C (PKC) activator 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) activated cell death in androgen-sensitive LNCaP cells but not in androgen-independent DU-145 or PC-3 cells, whose growth was significantly decreased by PKC inhibitors staurosporine and H7. All cell lines had similar levels of total PKC activities which, however, differed on their dependency on Ca^{2+} ions and lipid and were regulated differently by TPA. Furthermore, expression of the immediate early genes c-fos and c-jun was up-regulated by TPA only in LNCaP and DU-145 cells, whereas PC-3 cells failed to express c-fos mRNA. The regulation of the c-myc mRNA by TPA correlated inversely with activation of cell death being down-regulated in LNCaP cells, and slightly increased in the androgen-independent cell lines. These results suggest that the PKC signal transduction pathway functions differently in androgen-sensitive and insensitive prostatic cells.

Prostatic carcinoma is a complex disease whose etiology involves genetic changes (1), activated oncogenes and growth factors (2), androgenic hormones (3), as well as dietary factors. Advancing prostatic carcinoma is frequently associated with the loss of androgen (3). In some cases this failure has been associated with amplification of the androgen receptor gene (4), or with mutations in the androgen receptor that enable transcriptional activation by the binding of non-androgenic steroids (5). However, as androgen-independent prostatic cancer cell lines have lost the expression of the AR gene (6), other cellular pathways may be involved. Growth factors that are recognized to have an important role in prostatic tumor biology (2, 7, 8) signal through protein kinase C (PKC) (9-11) to elicits a plethora of cellular responses on cell growth and differentiation (12, 13). Furthermore, androgenic regulation of prostatic genes, including the androgen receptor, is disrupted when cells are treated with protein kinase C activators (14). Here we show that the PKC activator TPA elicits disparate responses in androgen-sensitive and insensitive prostatic cancer cells suggesting that alterations in PKC-isozymes may contribute to androgen insensitivity in prostatic cancer.

MATERIALS AND METHODS

Probes. The glyceraldehyde-3-phosphate dehydrogenase (GADPH) (15), the human epidermal growth factor receptor (EGFR) probe (16) and the c-myc probe (17) have been described previously. The c-fos probe was a 40-mer oligonucleotide complementary to nucleotides 1195-1235 of the second exon in the human c-fos gene (18) and the c-jun probe an oligonucleotide complementary to nucleotides 1036 - 1077 of the human c-jun gene (19). Standard methods were used to prepare the DNA plasmids used as probes (20).

Cell culture. LNCaP DU-145 and PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml) at 37°C in a humidified atmosphere with 5% CO_2. Control cultures were grown in RPMI-medium containing 10% charcoal (1%)-dextran (0.1%)-treated fetal calf serum (CSS), 2 mM glutamine and antibiotics and fungizone as above. All cell culture reagents were from Gibco, Paisley, UK. 17β-Hydroxy-17α-methyl-estra-4,9,11-trien-3-one (R1881) (New England Nuclear, Boston, MA) was dissolved in ethanol and 12-O-tetradecanoylphorbol-13-acetate (TPA), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), N-(2-guanidinoethyl)-5-isoquinoline-sulfonamide (HA 1004) and staurosporine (Sigma, St Louis, MO) in dimethyl sulfoxide before adding to culture medium. Triplicate cell counts were performed either with a hemocytometer, or by using a colorimetric method based on the reaction between cellular dehydrogenases and 3-[4,5-

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dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO).

Isolation and blot analysis of RNA. Total RNA from cultured cells was isolated (21) and RNA-blotting analyses and hybridizations were performed as has been described previously (22). All mRNA levels were corrected for loading errors by correlating the values obtained for specific mRNAs with the expression of GADPH mRNA in the sample which remained unaffected by the treatments described here.

Protein kinase C activity determinations. Cells were washed with 1 × PBS, and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 2 mM EDTA, 10 mM phenyl methyl sulfonyl fluoride, 25 mg leupeptin/ml, 25 mg aprotinin /ml, 1% Triton-X-100). The cell homogenate was centrifuged for 1 h at 100 000 × g, at 4°C. Aliquots of the supernatant were then used for the determination of protein kinase C activity according to the instructions provided with the Protein Kinase C assay system (Little Chalfont, UK). The activity of PKC was correlated to the amount of protein present in the sample.

RESULTS

Effects of PKC activator and inhibitors on the growth of prostatic cells. The phorbol ester TPA, is a well known activator of PKC (22). Within 10 minutes of treating LNCaP cells with medium containing 10 ng TPA/ml, their appearance changed from a spindle-like shape to a flattened shape with nuclei and membrane ruffles clearly visible. After the first 24 h, the number of LNCaP cells in plates which received medium containing TPA fell to 63 ± 0.07% of control (mean ± SE, $p < 0.001$) and within 48 h to 48 ± 0.06% of control ($p < 0.05$). During this time, the number of cells in the control cultures remained unchanged. Androgens are known to increase the growth of LNCaP cells (23, 24) but the synthetic androgen, R1881, could not prevent the TPA-induced apoptosis. The number of LNCaP cells fell to 58 ± 8% of control in 24 h to 50 ± 7% of control in 48 h in cultures treated with both TPA (10 ng/ml) and 0.1 nM R1881. In contrast to the effects on LNCap cells, treatment with TPA did not affect the appearance, nor the growth, of the androgen-insensitive prostatic cell lines DU-145 and PC-3. On the other hand, treating these cells with 0.1 μM staurosporine, which is a potent inhibitor of PKC (25), decreased cell numbers to 21 and 35 % of control in three days, respectively. H7, which affects the activity of both PKC and PKA (26), inhibited the growth of both DU-145 and PC-3 cells at high concentrations (50 and 100 μM), whereas HA 1004, which inhibits PKA (27), had no effect on the growth of these cells even at 100 μM concentration.

Analysis of PKC activity in LNCaP, DU-145 and PC-3 cells. LNCaP, DU-145 and PC-3 cells were cultured in medium containing 10 ng TPA/ml or in control medium. PKC activity was determined from total cellular lysates in the presence and absence of Ca^{2+}-ions and lipid. Total PKC activity did not differ markedly in extracts prepared from untreated DU-145, LNCaP and PC-3 cells, being 106.1, 75.6 and 89.6 pmol phosphate transferred/min/mg protein. However, whereas only 17% of the PKC activity in LNCaP cell extract showed dependency on Ca^{2+}-ions and lipid, 50% of PKC activity in DU-145 cells and 80% of that in PC-3 cells was dependent on their presence. Treatment of LNCaP cells with medium containing 10 ng TPA/ml resulted in a 2.7-fold increase in total PKC activity (expressed as pmol phosphate transferred/min/mg protein) within 1 h (Fig. 1). However, this increase was transient and after 3 h the activity in TPA-treated cells was only 130% of the control level, to which it returned within 24 h. In PC-3 cells, TPA-treatment resulted in a 1.3-fold increase within 30 minutes, followed by a decline to 80% of control within 3 h, at which level it remained, up to 24 h. In contrast, TPA-treatment of DU-145 cells resulted in decreased total PKC activity to 50% of control within 3 h and the decreased level persisted for the next 21 h.

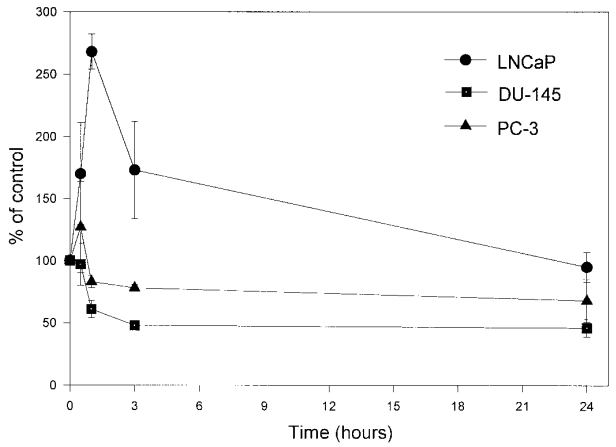

FIG. 1. The effect of TPA on the total cellular activity of PKC in prostatic tumor cells. DU-145, LNCaP and PC-3 cells were treated with medium containing 10 ng TPA/ml for different times up to 24 h. After the incubation, cells were harvested, and protein kinase C activity measurements were performed as described in Materials and Methods. Results for the TPA-treated cells are shown as % of the values obtained for untreated cells. Data is from 2 to 4 independent experiments and results for LNCaP cells are shown by black circles, those for DU-145 cells by black squares and those for PC-3 cells by black triangles.

The regulation of immediate early gene expression in LNCaP, DU-145 and PC-3 cells by TPA. In other cells, TPA has been shown to increase the expression levels of so-called primary response genes. These genes code for a variety of transcription factors and nuclear proteins, including the cellular proto-oncogenes c-fos, c-jun and c-myc (28). Expression of the c-fos gene was very low in uninduced LNCaP and DU-145 cells, but TPA-treatment up-regulated c-fos mRNA within 1 h, about 650-fold and 7-fold, respectively (Fig. 2). The increase in c-fos mRNA expression was transient in both cell lines, and after 24 h basal expression levels were reached. In contrast, TPA had no effect on c-fos mRNA

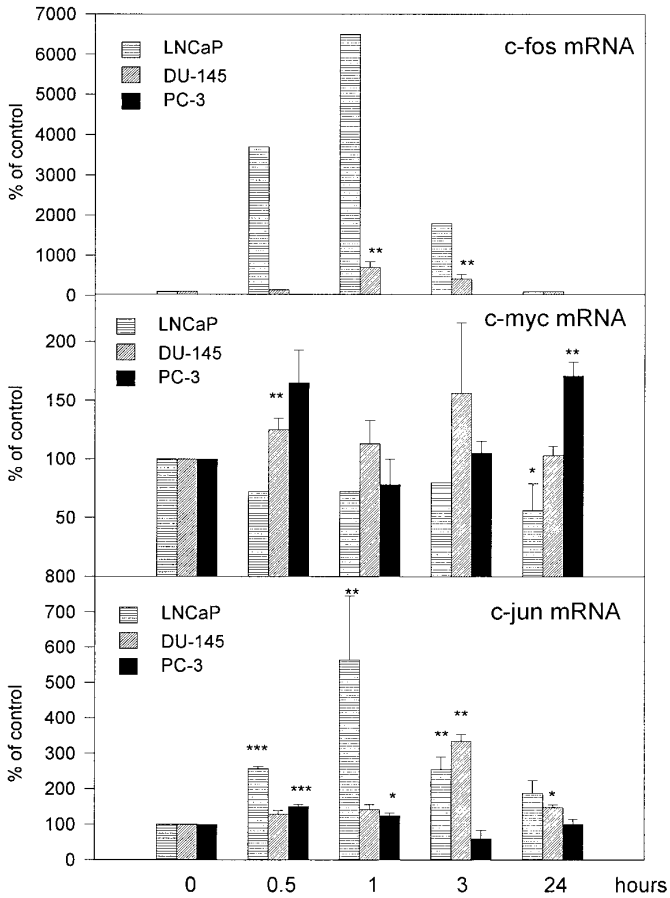

FIG. 2. TPA regulates immediate early gene expression differentially in DU-145, LNCaP and PC-3 cells. Cells were treated with medium containing 10 ng TPA/ml for the times indicated. Northern analysis was performed with c-fos, c-myc and c-jun probes. The values obtained by densitometric scanning of autoradiograms were correlated for the expression of GADPH mRNA in the sample and data is expressed as % of each mRNA level found in untreated samples. The data shown are means ± SE from 2-4 independent experiments. $P < 0.05$, **, $p < 0.01$ for treatment versus control.

in PC-3 cells, which failed to express this mRNA both before and after TPA-stimulation.

Untreated LNCaP cells contained about 7-fold more c-myc mRNA than either DU-145 or PC-3 cells. TPA-treatment of DU-145 cells resulted in a transient elevation of c-myc mRNA to 125 ±10%(SE) ($p < 0.01$) of control within 30 minutes and elevated levels were also detected after 1 h and 3 h, but by 24 h the level of c-myc mRNA had returned to control (Fig. 2). In TPA-treated PC-3 cells, the expression of c-myc mRNA was slightly elevated after 30 min but significantly increased levels 171 ± 12%(SE) of control ($p < 0.001$) were observed at 24 h. In contrast to the changes seen in the androgen-independent cells, the level of c-myc mRNA decreased in TPA-treated LNCaP cells to 72% of control after 1 h and to 56 ± 23%(SE) of control ($p < 0.05$) after 24 h (Fig. 2).

The highest basal level of c-jun mRNA was found in LNCaP cells, although it was expressed at very low levels in all three cell lines. The kinetics of c-jun mRNA induction by TPA were very different in the three cell lines studied here (Fig. 2). In LNCaP cells, TPA increased the steady-state level of c-jun mRNA significantly as soon as after 30 minutes to 257 + 5%(SE) of control ($p < 0.001$) and after 1 h the expression level peaked at 560 + 183%(SE) of control ($p < 0.05$). Elevated levels persisted also after 3 and 24 h. In DU-145 cells, c-jun mRNA responded more slowly to TPA but was up-regulated to 328 ± 20%(SE) of control ($p < 0.001$) after 3 h, and it continued to be increased for up to 24 h. In PC-3 cells, c-jun mRNA was only modestly up-regulated by TPA-treatment, to 148 ± 4%(SE) of control ($p < 0.001$) in 30 minutes and to 122 ± 6%(SE) of control ($p < 0.05$) at 1 h.

The regulation of immediate early gene expression in LNCaP, DU-145 and PC-3 cells by EGF. Epidermal growth factor promotes the growth of LNCaP cells but not that of DU-145, PC-3 cells (2, 29) and it also regulates immediate-early gene expression (30). Therefore, it was of interest to study the effects of 10 ng/ml EGF on the expression of c-fos, c-jun and c-myc genes in prostatic cells. In LNCaP cells the levels of all three mRNAs were upregulated within 30 minutes of administering EGF to the cells (Fig. 3.). The levels of all the three mRNAs were also increased in DU-145 cells, although to lower levels than in LNCaP cells. In contrast, no significant changes could be observed in PC-3 cells and the expression of the c-fos gene was also refractive to EGF in PC-3 cells.

DISCUSSION

In this study, we show that the involvement of the PKC pathway in cell growth, enzyme activities and gene induction is different in androgen-sensitive and insensitive prostatic cells. Firstly, phorbol ester, TPA, a potent activator of protein kinase C, triggered cell death only in androgen-sensitive prostatic cancer cells. On the other hand, inhibitors of PKC but not that of protein kinase A prevented proliferation of androgen-independent prostatic tumor cells, implying that cellular PKC-activity was contributing to their faster growth rates.

Secondly, the PKC enzyme activity found in LNCaP cells was less dependent on Ca^{2+}-ions and lipid than and it was also increased by TPA-treatment unlike the activities found in DU-145 or PC-3 cells, thus suggesting that the PKC isoforms expressed in these cells are partially different. The Ca^{2+}-dependent PKC isoforms comprise PKC-α, β and γ, whereas the other isoforms, the novel PKCs, demonstrate activity without Ca^{2+}-ions (10). Thus, the low proportion of Ca^{2+}-dependent activity (17% of total PKC activity) in LNCaP cells

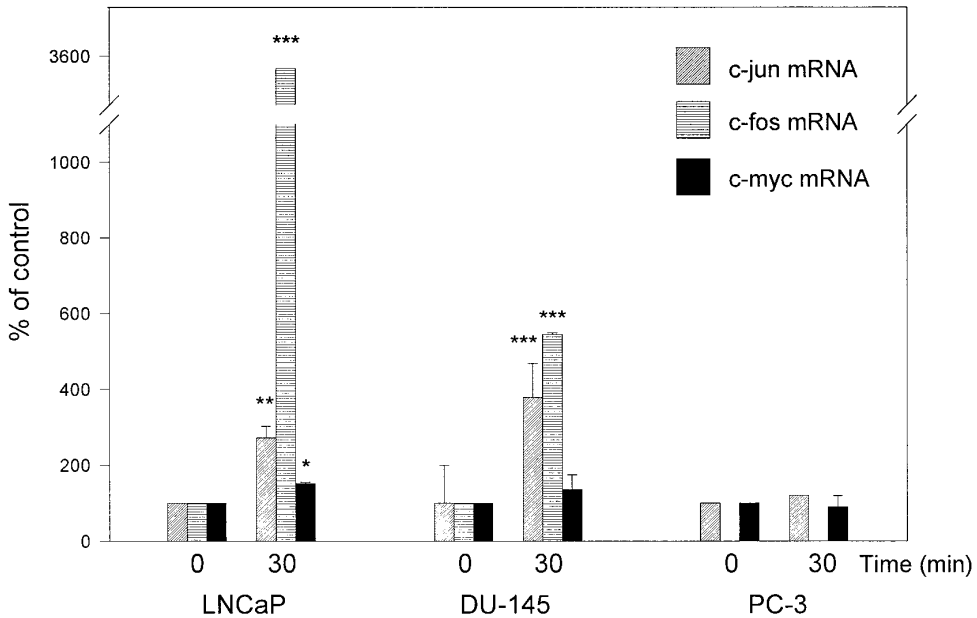

FIG. 3. Regulation of immediate early gene expression by EGF. Cells were treated with medium containing 10 ng/ml EGF for 30 minutes. Total RNA was isolated from cell lysates and Northern analysis was performed as detailed in Materials and Methods. The data are given as % of the value found in untreated cells. The data is from 3 independent experiments and means ± SE are given. $P < 0.05$, **, $p < 0.01$ for treatment versus control.

may indicate a higher expression level of novel PKC isoforms than in either DU-145 or PC-3 cells. Previously, it has been reported that normal rat prostate and Dunning R-3327 rat prostatic adenocarcinoma sub-lines express mRNAs coding for at least 8 different PKC isoforms. Of these transcripts, those coding for PKC-α were expressed at a 4-fold higher level in the tumor cell lines than in normal prostate, whereas the androgen-insensitive cell lines contained less PKC-ξ mRNA than normal prostate or the androgen-sensitive sub-lines (30). While this manuscript was in preparation, Powell and associates (31) reported that LNCaP cells express less PKC-α and more PKC-μ than the androgen-insensitive DU-145 and PC-3 cell lines.

Thirdly, the regulation of immediate early gene expression by TPA was dissimilar between androgen-sensitive and androgen-independent cell lines. Whereas TPA-treatment elicited a rapid and pronounced increase in both c-jun and c-fos gene expression in LNCaP cells, increases in the levels of these mRNAs were more modest and had a different time-course in DU-145 cells. Interestingly, PC-3 cells, whose growth is totally refractive to TPA, show no expression of the c-fos gene and very limited responses in expression of the c-jun gene. Krongrad and Bai (32) have similarly observed the expression of the c-fos gene is not responsive to TPA that in other androgen-independent prostatic tumor cells. It has previously been shown that TPA-mediated apoptosis of androgen-sensitive prostatic cells is accompanied by increased expression of c-fos (33). However, as EGF, which is mitogenic to LNCaP cells and does not activate apoptosis, up-regulates both c-fos and c-jun expression, it is difficult to visualise a critical role for either gene product in apoptosis. On the other hand, expression of the c-myc gene closely parallels the fate of LNCaP cells. Agents that cause the death of LNCaP cells, such as TPA and cycloheximide (34), decrease the amount of c-myc mRNA (Fig. 2. and our unpublished observations), whereas growth-promoting compounds, such as EGF, increase it (Fig. 3.). Notably, in the androgen-insensitive cell lines, c-myc expression is not decreased by TPA, or cycloheximide, neither of which agents causes cell death in these cell lines (Fig. 2 and our unpublished observations). Previously, the c-myc gene has been implicated as a candidate for a molecular switch whose expression level is critical in controlling cell growth and death (35), but its precise role remains to be clarified. Our results show that it may also have an important role in the regulation of prostatic apoptosis.

Taken together, these findings show that the PKC pathway functions differently in androgen-dependent and independent prostatic cell lines. Furthermore, changes in the expression of PKC isoenzymes may be involved in the loss of androgen-sensitive phenotype in prostatic tumor cells. We have previously shown that activation of PKC by TPA results in down-regulation of expression of genes coding for prostatic-secretory proteins and this down-regulation cannot be reversed by androgens (14). Here we showed that the proliferation of TPA-treated cells was no longer affected by androgens. In effect, TPA-treatment renders previously

androgen-sensitive cells refractive to androgens, a phenomenon frequently associated with prostatic cancer. Furthermore, studies with breast carcinoma cells have shown that over-expression of the PKC-δ isoform results in the loss of estrogen-sensitive growth and gene regulation. This is accompanied by altered expression of several other PKC isoforms, including PKC-α, in the transfected cells (36). Changes in the expression of PKC-isoforms that occur in advancing prostatic carcinoma may contribute to the loss of androgen-regulated gene expression by favoring the expression of those isoforms that repress prostate-specific and androgen-regulated gene expression. Further research into the function of signal transduction pathways in prostatic cancer cells may provide us with the keys to understanding the biology of prostatic malignancies.

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