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FULL LENGTH ARTICLE

Forskolin and Phorbol 12-myristate 13-acetate modulates the expression pattern of AP-1 factors and cell cycle regulators in estrogen-responsive MCF-7 cells

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Abstract Activator protein-1 (AP-1) transcription factor is a key component of many signal transduction pathways involved in the regulation of cellular processes and controls rapid responses of mammalian cells when exposed to the variety of stimulus. The phorbol 12-myristate 13-acetate and Forskolin (Fo) are well-known kinase activators/stimulators of Protein Kinase C (PKC) and Protein Kinase A (PKA) respectively. Importantly, these kinases are found to be present in transitional points of many cell signaling pathways, especially those involved in proliferation. The stimulating effect of PKC and PKA on the expression of AP-1 factors in MCF-7 breast cell proliferation is not well characterized. Hence, the role of PKC by PMA treatment and the role of PKA by using Fo in MCF-7 cells is investigated. Where, cells treated with PMA showed increased cell proliferation, while Fo had no effect, but inhibited the PMA induced proliferation. The RT-PCR results showed the PMA induced c-Jun, c-Fos and Fra-1 expressions compared to control and Fo. However, Fo in combination with PMA, inhibit the PMA induced above mRNA expressions where Fo alone has no effect. Western blot studies validated

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the c-Jun expressions in PMA treated MCF-7 cells. Further, PMA increases the mRNA expression of Cyclin-E1, Cyclin-D1, and CDK-4, whereas Fo decreases their expressions. Thus, mitogenic effect of PMA and inhibitory action of Fo on MCF-7 cells is probably enhanced via activation of AP-1 factors and concomitant action of cell cycle regulators in the downstream singling cascade.

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Introduction

Breast cancer is the most common malignancy found among women in the world, with constant rise in the developing nations.[1] Breast cancer development is a complex process and involves several proteins, hormones, enzymes that regulate tumor progression and survival.[2] Enzymes like PKC and PKA are involved at transitional points of many cellular pathways regulating various cellular process and proliferation as well. Earlier studies report the presence of elevated levels of PKC isoforms during proliferation, apoptosis and anti-estrogen resistant mammary tumor cells.[3,4] Similarly, elevated activity of PKA has been found in cell proliferation in many types of cells,[5,6] including MCF-7 cells.[7] PKC and PKA signaling pathways are also involved in the activation of proto-oncogenes and subunits of AP-1 factors to regulate several cellular processes.[8,9] However, independent or synergistic effect of PKC and PKA on MCF-7 cell proliferation and their coordinated role on expression pattern of AP-1 factors is poorly understood. Hence, the present study was carried out to understand the role of PKC and PKA on the expression pattern of AP-1 during MCF-7 cell proliferation.

PKC, a multigene super family of serine/threonine kinase and its isoforms are implicated in a wide range of G protein-coupled receptors mediated and other growth factor-dependent cellular responses.[10] Many PKCs are pharmacologically activated by tumor-promoting phorbol esters such as PMA,[11] which anchors PKC in their active conformation to membranes. Based on the differences in N-terminal regulatory domain, PKC isoforms are subdivided into conventional PKCs, novel PKCs and atypical PKCs which are specifically activated by different stimuli. Because of the existence of multiple PKC isoforms, many mitogenic signaling cascades operate during various cellular processes and may converge at a distal point, possibly causing the activation of an AP-1 factor that can then exert a specific biological effect. Hence, understanding the coordinated role of PKC and AP-1 factors may reveal their influence on mitogenic mechanisms in breast cancer cells. In addition, the study also helps to understand the synergistic effect of PKC and PKA pathways in MCF-7 cells.

PKA is a cAMP dependent protein kinase, through various stimuli cAMP activates PKA and brings the desired effects in cells. Over twenty years ago, various research groups, working with different cancer cell types, established the role of cAMP in cell cycle growth and cancer development.[12] The cAMP seems to act as an activator as well as an inhibitor of cell proliferation. Hormonal cAMP regulation of the ERK cascade provides an important crosstalk between hormones and growth factor signaling.[13] Hormones that stimulate adenyl cyclase induce the production of cAMP which in turn activates or inhibits ERK in a cell specific manner. Thus, multiple pathways exist for cAMP to either inhibit or activate ERK signaling via PKA-dependent and independent pathways. Many laboratories reported that cAMP inhibits the cell growth of adipocytes,[14] endothelial cells,[15] NIH 3T3 cells[16] by blocking growth factor activation of ERKs. The cAMP has also been shown to activate multiple intracellular signaling cascades independent of its activation of PKA,[17] however, most of the studies examining cAMP inhibition of ERKs show a requirement for PKA.[14–16] The above conflicting findings prompted us to study the role of cAMP in the proliferation of MCF-7 cells and its effect on AP-1 factors. MCF-7 cells were used as model system in this study, since MCF-7 is an estrogen responsive cell line and widely used as model system to understand the molecular mechanism underlying in Breast cancer.

In an earlier study using granulosa cells, Sharma et al, (2000) showed synergistic action of Fo and PMA in enhancing transcription of an AP-1 luciferase construct (−73 col) mediated through Jun-D clearly suggest the involvement of PKC and PKA in regulation of AP-1 factor.[18] Our recent study using E2 on expression pattern of AP-1 suggested that c-Jun, c-Fos and Fra-1 were rapidly induced, while anti-estrogen Tamoxifen (TMX) decreased the expression.[19] In our study we used Fo, a diterpene which indirectly activates the PKA via cAMP signaling. Other mitogens like 1, 2 diacyl glycerol (DAG) or hormones that increase intracellular cAMP may have similar effects on the expression pattern of AP-1 factors. However, less is known about the role of PKA and PKC in the signal transduction pathways that regulate the expression pattern of AP-1 factors in cells.

AP-1 factors are implicated in the regulation of cell proliferation, migration, inflammation, apoptosis and many more cellular processes.[19–21] The Jun (c-Jun, Jun-B, and Jun-D) and Fos (c-Fos, FosB, Fra-1 and Fra-2) are the major AP-1 proteins involved in gene modulation.[22] Jun proteins can be both homo and hetero-dimerize, whereas Fos proteins cannot homo-dimerize, but do hetero-dimerize with Jun proteins. The activated AP-1 factors that are dimers, Jun/Jun or Jun/Fos, bind to their cognate DNA sequences of target genes called as 12-O tetradecanoylphorbol-13-acetate (TPA) response element 5′-TGA (C/G) TCA-3’ (TRE) that activates transcription in the regulatory regions of target genes.[23] Substantial studies have shown PKC and PKA to activate AP-1 factors when exposed to external stimuli.[24,25] However, precise AP-1 gene expression patterns are yet to be investigated on activation of PKC and PKA in MCF-7 cells. We also studied how the PKC and PKA bring changes on apoptosis and cell cycle regulator genes.

In the present study, we have investigated the role of cAMP, a second messenger involved in the PKA activation, using Forskolin (Fo) an activator of adenyl cyclase, and the role of PKC pathway which is activated using the mitogen Phorbol ester, an analogue of diacyl glycerol (DAG), and further status of MCF-7 cell proliferation and expression pattern of AP-1 factors and cell cycle regulators was elucidated using RT-PCR and Western blotting methods.

Materials and methods

Materials

Human breast cancer cells (MCF-7) were purchased from NCCS (Pune, India), Phorbol-12-myristate-13-acetate and forskolin (Fo) were purchased from Sigma–Aldrich (St Louis, USA). Fetal bovine serum (FBS), penicillin, streptomycin, glutamine, RPMI 1640 media, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Phosphate Buffer Saline (PBS), Bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from Himedia (Mumbai, India). Oligo-dTs and super script reverse transcriptase were procured from Invitrogen Bio-Services India Pvt. Ltd (Bengaluru, India). Taq DNA polymerase (1 U/μl) was purchased from Merck-Millipore (Mumbai, India). Forward and reverse oligo primers for different AP-1 factors, cell cycle regulators and apoptosis gene sequences were designed (Table 1) and were purchased from Sigma–Aldrich (St Louis, USA). Antibodies and HRP conjugate substrate were purchased from Imgenex India Pvt. Ltd. (Bhubaneswar, India) and Merck-Millipore (Mumbai, India).

Culturing of cells

MCF-7 cells (human breast cancer cells) were cultured in RPMI 1640 medium supplemented with or without serum (FBS), penicillin (100u/ml) and streptomycin (100u/ml) under the atmosphere of 5% CO_2, 95% air in humidified incubator at 37 °C. The 25 cm^2 culture flask containing 80–90% confluent cells were sub cultured in to 96-well plate (3×10^3 cells/well) or 6-well plate (3×10^5 cells/well) for the experimental studies.

Cell viability assay

Cell viability assay was carried out using MTT as per the protocol described earlier[26] with slight modifications. In brief, MCF-7 cells (3×10^3 cells/well) in RPMI-1640 medium with a final volume of 200 μl were seeded into 96-wells plate and cultured overnight at 37 °C in a CO_2 incubator. The cells were serum starved before the treatments and incubated for 48 h with or without PMA or Fo. Further, following a wash with PBS, the cells were treated with 20 μl of MTT (5 mg/ml) alone and incubated for 4 h at 37 °C in a CO_2 incubator. Blue formazan products formed in cells were dissolved in DMSO (100 μl) and spectrophotometrically measured at 540 nm. The proliferative effect of PMA or Fo, or PMA and Fo treated cells were calculated and represented as a bar diagram.

RNA isolation and RT-PCR analysis

The overnight cultured MCF-7 cells in 6-wells plate were serum starved and treated with or without PMA (10 nM) or Fo (10 μM) or PMA + Fo for 48 h. Total RNA was isolated from control and treated MCF-7 cells using "TRIzol" reagent as per the manufacturer's instructions. Reverse transcription and PCR analysis were carried out as described earlier (Babu, et al, 2013). In brief, 2 μg of total RNA of different samples were reverse transcribed using oligo-dT primers and Superscript reverse transcriptase. First-strand cDNA (1 μl) was mixed with 2 μl of 10X PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 1 μl 50 mM $MgCl_2$, 1 μl 10 mM dNTP's and 1 unit Taq DNA polymerase was added to a 20 μl final reaction volume. The cDNA was subjected to 30 cycles of PCR in a gradient Eppendorf thermocycler using different forward and reverse primers of AP-1 factor genes, cell cycle regulators or of apoptosis with an specific annealing temperatures as in Table 1. Expression of β-actin was used as a positive control and for normalization. Amplified PCR products were analyzed on 1% agarose gel electrophoresis using 1X TAE Buffer. Relative mRNA levels were quantified using image analysis software (ImageJ-https://imagej.nih.gov/ij/). The expression of β-actin mRNA was used as a positive control and for normalization.

Western blotting

Total cell protein extracts of MCF-7 cells were prepared and analyzed by western blotting. The control and treated cells containing culture plates were placed on ice for 10 min, washed thrice with PBS (1X) and 0.5 ml of lysis buffer was added to each well and incubated for 5 min at room temperature. Followed by scrapping, the cells were transferred to pre-cooled 1.5 ml centrifuge tube, mixed gently by swirling on ice and centrifuged at 15,000×*g* for 20 min at 4 °C. Supernatant was transferred to fresh tubes and used for analysis. Protein concentration was determined by the Bradford method.[27] Equal protein concentrations (50 μg) of samples were separated on SDS-polyacrylamide gel electrophoresis (PAGE). Proteins present in SDS-PAGE were electrophoretically transferred to a PVDF membrane. The membrane was blocked with 5% Carnation fat free milk at room temperature for 1 h. Antibody to c-Jun (1:1000) was added in the same blocking solutions and incubated further for 1 h. The membrane was washed and incubated with anti-rabbit antibody-HRP (1:500) for 1 h at RT. Immunoreactive proteins were visualized using Luminata forte western HRP substrate and analyzed as per the specifications provided by the supplier. Immunoreactive bands were quantified using image analysis software (ImageJ-https://imagej.nih.gov/ij/). GAPDH was used as a positive control and for normalization.[28]

Statistical analysis

The experimental data are shown as mean ± standard deviation from three independent experiments. Statistical analyses were done by Student's t-test and one-way ANOVA followed by post hoc tukey test. Values were considered statistically significant at $^{*}P < 0.05$ compared to control and $^{\#}P < 0.05$ when compared to PMA treated sample.

Table 1 Sequence of primers used in the RT-PCR studies.

Gene	Primer Sequence (5′→3′)	Annealing temp. (°C)	Product size (bp)
ERα	F: TACTGCATCAGATCCAAGGG R: ATCAATGGTGCACTGGTTGG	61	650
ERβ	F: TGAAAAGGAAGGTTAGTGGGAACC R: TGGTCAGGGACATCATCATGG	60	528
PKCε	F: CTTCTCGACCCCTACATTGCC R: GCAGGTGCAGACTTGACACTG	61	449
c-Jun	F: GCCTACAGATGAACTCTTTCTGGC R: CCTGAAACATCGCACTATCCTTTG	64	525
Jun-D	F: CGCAGCCTCAAACCCTGCCTTTCC R: AAACAGGAATGTGGACTCGTAG	64	500
Jun- B	F: CCAGTCCTTCCACCTCGACGTTTACAAG R: GACTAAGTGCGTGTTTCTTTTCCACAGTAC	58	257
c-Fos	F: TCTTCCTTCGTCTTCACC R: AATCAGAACACACTATTGCC	58	577
Fra-1	F: AGGAAGGAACTGACCGAC R: GAAGGGGAGGAGACATTG	60	497
Fra-2	F: AGGAGGAGAGATGAGCAG R: GGATAGGTGAAGACGAGG	60	518
Fos-B	F: TGTCCCAGGGAAATGTTTCAGGC R: ACTGGTAGTTCCGCTGGTGGAAGG	56	451
p53	F: GAGCCCCCTCTGAGTCAG R: GCAAAAACATCTTGTTGAG	56	375
CDK4	F: AGTGGCGGATCCATGGCTACCTCTCGATAT R: TCTCGGAAGCTTTCACTCCGGATTACCTTCA	60	912
Cyclin D1	F: AGACCTGCGCGCCCTCGGTG R: GTAGTAGGACAGGAAGTTGTTG	58	574
Cyclin E1	F: GTCCTGGCTGAATGTATACATGC R: CCCTATTTTGTTCAGACAACAT	60	415
Bcl-2	F: AGATGTCCAGCCAGCTGCACCTGAC R: AGATAGGCACCCAGGGTGATGCAAGCT	62	365
Bax	F: AAGCTGAGCGAGTGTCTCAAGCGC R: TCCCGCCACAAAGATGGTCACG	61	366
β-actin	F: TACCACTGGCATCGTGATGGACT R: TCCTTCTGCATCCTGTCGGCAAT	62	516

Columns 3 and 4 show annealing temperatures and the size of the amplified products. **F** forward, **R** reverse.

Results

Forskolin inhibits PMA induced MCF-7 cell proliferation

As described in the methods, the effect of PMA/Fo on proliferation of MCF-7 was carried out using the MTT assay. MCF-7 cells treated with PMA (10 nM) showed significant increase in cell proliferation (32%) compared to control, with $P < 0.05$ (Fig. 1). However, Fo a protein kinase A activator, did not show any effect in terms of proliferation, while the PMA + Fo compared to PMA alone showed a slight inhibition of proliferation of MCF-7 cells.

PMA and forskolin regulates the mRNA expressions of estrogen receptors, PKCε and cell cycle regulators

Expression analysis of genes involved in cell cycle regulators and apoptosis process, suggested that the MCF-7 cells

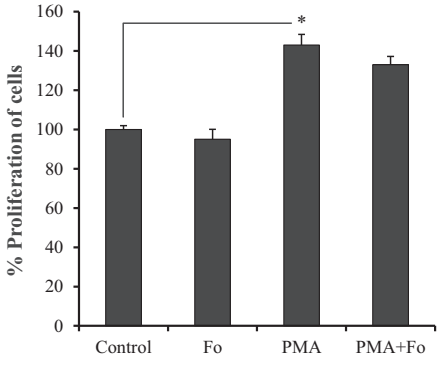

Figure 1 Effects of PMA and Forskolin on the MCF-7 cells proliferation. MCF-7 cells were treated with or without PMA (10 nM) or Fo (10 μM) or PMA + Fo and cell proliferation was determined by MTT assay. Results were expressed as relative proliferative index (mean ± SD, n = 8). Values were significantly different from control at *P < 0.05. The results were shown as representative of three independent experiments.

express transcripts at different levels pertaining to cells cycle regulators (p53, Cyclin D1, Cyclin E and CDK4), apoptotic genes (Bax and Bcl2), ERs (ERα and low amounts of ERβ) and PKCε. Fo showed a slight inhibitory effect on the expression of Cyclin D1, p53, CDK4 and anti-apoptotic Bcl2, ERα, ERβ and, PKCε. While PMA had little or no effect on the above cited genes, it did have a significant stimulatory effect on Cyclin E1, and a marginal effect on CDK4 expression. Fo also showed decreased effect on CDK4 expression induced by PMA. However, both Fo and PMA have no effect on pro-apoptotic Bax mRNA levels (Fig. 2a and b).

Forskolin inhibits PMA induced AP-1 factors expressions in MCF-7 cells

To analyse the role of PKC and PKA on the expression of mRNAs of AP-1 factors, the MCF-7 cells were treated with and without PMA (10 nM) or Fo (10 μM). In MCF-7 cells treated with PMA, the Jun family (c-Jun, Jun-B and Jun-D) and Fos family (Fra-1, Fra-2 and c-Fos) mRNA transcripts were present and expressed at different levels. PMA induce the c-Jun, c-Fos and Fra-1 transcripts significantly compared to control (Fig. 3a and b). However, Fo decreased the expression of c-Jun. Fra-1 mRNA levels were present at low levels in control and Fo has no effect but decreased the PMA induced Fra-1 expression and also the c-Fos mRNA levels. Fos-B mRNA transcript was present in very low levels or absent and found to be unaffected by Fo and PMA (data not shown). Western blot analysis also show that Fo significantly decreased c-Jun protein levels, while a 20% increase was seen with the treatment of PMA. Fo was also found to decrease PMA induced c-Jun as well as basal level (Fig. 3c).

Discussion

In the present study, stimulated effect of PKC and PKA on the proliferation, cell cycle regulation and the involvement of AP-1 factors was investigated using human breast cancer MCF-7 cells. The cells treated with PMA increased the proliferation but Fo was found to have no effect compared to control. PMA induced the expression of c-Jun, c-Fos and Fra-1 mRNAs to exactly the same degree shown by E2 as previously reported,[19] while Fo action was unaffected by PMA. These findings suggest the involvement of c-Jun/c-Fos AP-1 factors in MCF-7 cell proliferation and transformation. Studies carried out using MCF-7 cells, found that IGF1 induced c-Jun, c-Fos and Fra-1 mRNAs, while Jun-B, Jun-D, Fos-B and Fra-2 were only expressed at low levels, thus confirming the role of specific AP-1 factors in proliferation.[29] The basic mechanism involved in growth factor signaling includes receptor binding and activation of cytoplasmic signal transduction cascade resulting in the phosphorylation of AP-1 (c-Jun/c-Fos) dimers. Activated c-Jun/c-Fos induces AP-1 dependent genes. The present study logically shows that PKC employs a mitogenic effect that is similar to that of estrogen by inducing similar patterns of AP-1 factors in MCF-7 cells.

The experimental data shows almost negligible quantities of Fra-1 mRNAs present in control MCF-7 cells;

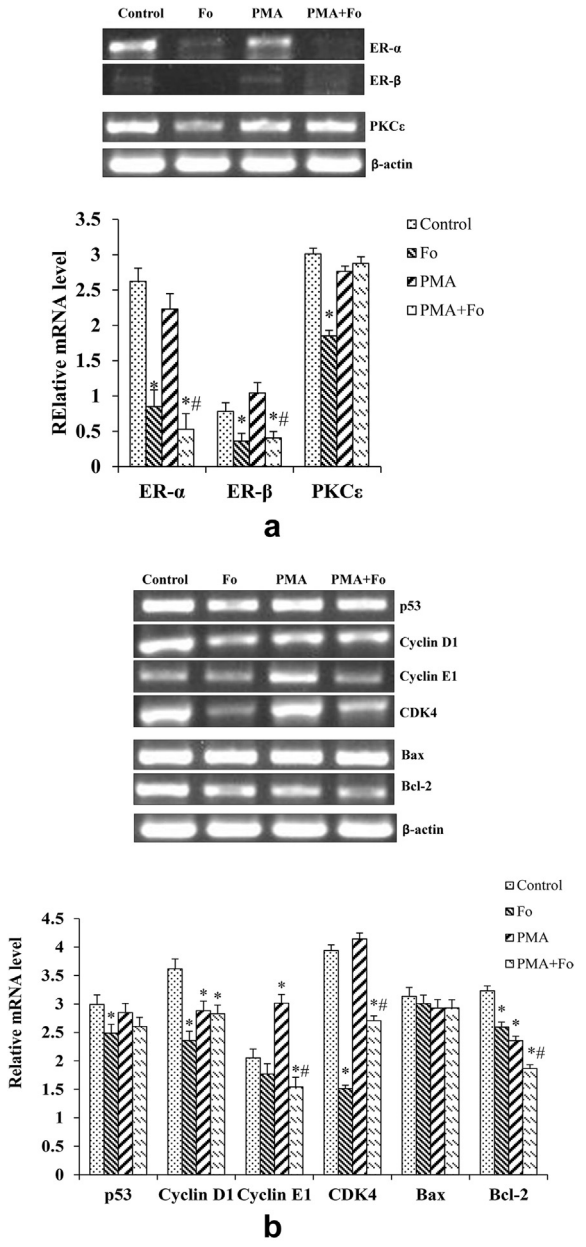

Figure 2 Effect of Fo/PMA on a) ERs and PKCε, b) cell cycle regulators and apoptosis mRNA transcripts. MCF-7 cells cultured in 6-wells plate were treated with or without Fo (10 μM) or PMA (10 nM) or PMA + Fo for 48 h in 6-wells plate. Total RNA was extracted and cDNA was prepared and subjected to 30 cycles of PCR amplication using specific primers of cell cycle regulators, apoptosis, ERs and PKCε genes and β-actin (positive control). The mRNAs expression were analyzed on agarose gel (1%) and statistically significant: if *P $<$ 0.05 compared with controls, #P $<$ 0.05 compared with PMA treated by post hoc Tukey test. The bar graph represents the densitometric analysis of respective mRNA levels.

however, the cells treated with PMA experienced induced mRNA transcription levels by several folds. Fra-1 mRNA levels were unaffected by Fo treatment and also did not show any changes in expression levels in PMA induced MCF-7 cells. Our experimental data on MCF-7 cells points out

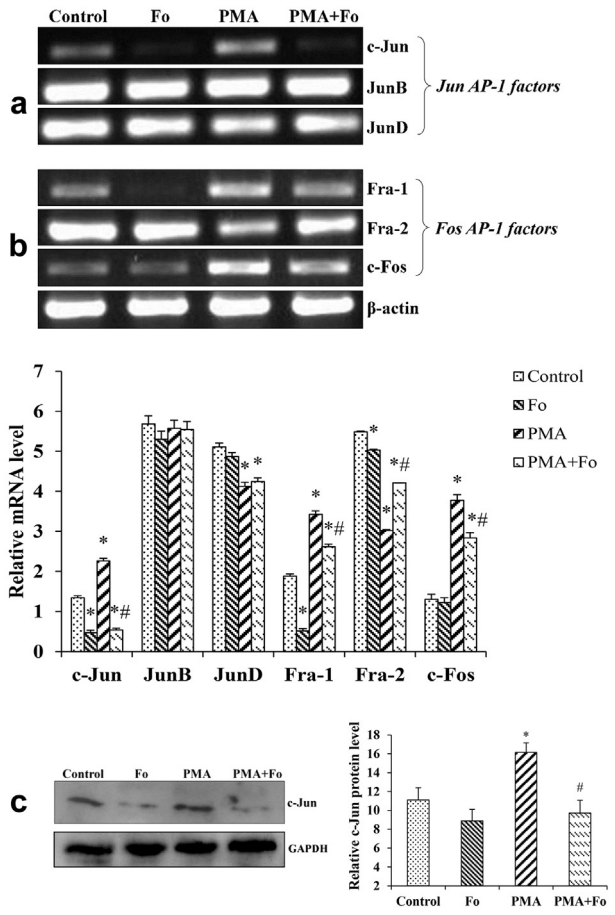

Figure 3 Expression pattern of mRNAs of AP-1 factors (a) Jun family (b) Fos family in MCF-7 cells and (c) c-Jun protein levels. MCF-7 cells were treated with Fo (10 μM) or PMA (10 nM) or PMA + Fofor 48 h in 6-wells plate. Total RNA was extracted and cDNA was prepared and subjected to 30 cycles of PCR amplification using desired primers of Jun and Fos family members and β-actin (positive control). The mRNAs expression were analyzed on agarose gel (1%). For Fig. 2c total protein was extracted and c-Jun was analyzed using specific antibodies by Western blotting. Data expressed are mean ± SD from triplicate experiment. Differences in AP-1 factors mRNA expression are considered significant: if *P < 0.05 compared with controls, #P < 0.05 compared with PMA treated by post hoc Tukey test. The bar graph represents the densitometric analysis of mRNA levels of AP-1 factors and c-Jun Protein levels.

that, like E2, PMA induces Fra-1 mRNA transcripts, and that, an anti-estrogen, inhibits these transcripts,[19] however, PMA had no effect on the related Fra-2. Fo marginally inhibited the PMA induced Fra-2 expression. Both Fra-1 and Fra-2, lack c-terminal transactivating domain characteristic of c-Fos/Fos-B and hence does not stimulate AP-1 responsive promoters under *in vitro* conditions.[30–32] Probably PMA induced Fra-1 in MCF-7 cells may dimerize with c-Jun inducing the proliferation and metastasis. More experiments like the electro mobility shift assays may help to elucidate the role of Fra-1 in proliferation. Fos-B mRNA was weakly expressed or undetectable in MCF-7 cells (data not shown). This observation was in agreement with a previous study showed that Fos-B was down regulated in human breast carcinoma cells when compared to normal mammary epithelial cells.[33] This might be due to instability of the Fos-B transcripts, having a reported half-life of 10—15 min, a finding which is a typical characteristic for immediate and early genes.[34]

Although PMA showed a marginal effect on Cyclin D1 expression, significant increase in Cyclin E1 and marginal increase of CDK4 transcripts suggested that PKC activate cell cycle regulators and mitogens are responding through involvement of AP-1 factors. Studies also reported a similar kind of regulation wherein CAGE-induced expression of cyclin D1 and E was found to be mediated by AP-1 and E2F-1 transcription and their by upregulate the cancer cell proliferation.[35] Among the AP-1 c-Fos may be an important factor in the mitogenic response of E2/PKC, as several genes (E2 F1, SKP-2, CDC-25A, BCL-2, AREG, CDC14A etc.) of cell cycle, DNA replication and transcription were upregulated.[36] The importance of AP-1 factors in regulating breast cancer cell growth has been documented.[37] Estrogen and c-Fos have been found to regulate E2F1 pathway.[38] Where, the c-Fos is also involved in estrogen induction of E2F1 expression through a mechanism potentially involving ERα and AP-1 recruitment to the E2F1 promoter. E2F1 transcriptional targets regulated by ER-α/c-Fos dependent mechanism/c-Fos independent mechanisms have been shown to participate in regulating the cell cycle, apoptosis, and cellular proliferation and differentiation processes. More studies are required on the interaction of AP-1 and E2F1 transcriptional factors as they probably involved in cyclin—cyclin dependent kinases/retinoblastoma pathways and the regulation of the expression of numerous genes involved in replication and cell cycle progression.[39]

In MCF-7 cells Fo significantly decreased the PMA induced c-Jun and c-Fos expression, while marginally decreasing Fra-2 expression. These findings suggest that PKA has an inhibitory effect on the expression on specific AP-1 factors. In addition, Fo also significantly decreased the mRNA transcripts of cyclin D1, CDK4, ERα, while marginally decreasing the expression of cyclin E1 and PKC-ε. This evidence suggests that Fo, the activator of PKA, has antiproliferative effect in MCF-7 cells. Further, Fo decreased the PMA induced proliferation of MCF-7 cells. Similar results were reported with thyroid follicles,[40] rat glioma cells,[41] where c-Fos and c-Jun expression were induced by phorbol ester or activation of protein tyrosine kinase, but inhibited by Fo an activator of PKA. Studies carried out in rat adipocytes or CHO cells suggested that MAP kinase was attenuated by Fo or 8Br cAMP, or glucagon.[14] Similar decreases in the activity of MAP kinase by PKA may possibly occur in MCF-7 cells and it is interesting to elucidate the exact steps at which the Fo inhibits the MAP kinase pathway. Further, the mitogenic effect of PKC and its inhibition by PKA is probably mediated via activation of AP-1 factors (c-Jun, c-Fos and Fra-1) along with controlled cell cycle regulators expression (cyclin E1 and CDK-4) in human breast cancer MCF-7 cells. Thus, the study provides an input in elucidating the role of PKC/PKA signaling cascade during cell proliferation and these findings may help in designing effective therapeutic strategies for the treatment of breast cancer.

Conflict of interest

The authors have no conflict of interests to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2018.12.001.

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