J. Biosci., Vol. 21, Number 4, June 1996, pp 477-485. © Printed in India.

Insights into mechanism of tumour promotion by mezerein

NANDINI RUDRA, NEETA SINGH† and MALAYA GUPTA*
Department of Biochemistry, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, 110029, India
*Department of Pharmaceutical Technology, Jadavpur University, Calcutta 700032, India

MS received 24 July 1995; revised 10 January 1996

Abstract. The objective of this study was to gather insights and compare the mode of action of the non phorbol, diterpene mezerein with the phorbol ester, phorbol-12-myristate-13 acetate, in normal and transformed cells. Both phorbol-12-myristate-13 acetate and mezerein are shown to activate the signal transduction pathways involving post translational modification of proteins by poly ADP-ribosylation and by protein kinase C, but to varying extents and showed different time kinetics and cell type differences. Multiple nuclear proteins, especially histones H3d, A24 and HI served as acceptors of poly ADP-ribose in response to PMA in both NIH 3T3 and HDCS cells whereas H1 and H2B were the major acceptors in case of mezerein treatment, similarly in both NIH 3T3 and HDCS cells. The results suggest an epigenetic mechanism (s) in tumour promotion by mezerein.

Keywords. Tumour promotion; mezerein; ADP-ribosylation; protein kinase C.

1. Introduction

Chemical carcinogenesis is well defined as a multistep process in mouse skin model with tumour promotion as an essential component (DiGiovanni 1992). Tumour promoters lead to altered gene expression and chromatin structure as well as disturbances in signal transduction pathways (Cerutti 1985). The potent phorbol ester tumour promoter phorbol-12-myristate-13-acetate (PMA) is a membrane acting agent and mediates part of its action through active oxygen species (Sun 1990; Birnboim 1986). Mezerein a diterpene, is comparable in efficacy to the more potent phorbol ester PMA as an inflammatory agent but is 50 times less potent as a complete tumour promoter (Fibac *et al* 1984). PMA and mezerein have been shown to share common biological and biochemical effects in mouse skin (Mufron *et al* 1979). The present study was undertaken to explore how mezerein exerts its tumour promoting effect in comparison to PMA via post translational signal transduction mechanism(s) in both normal and transformed cells. Mezerein was found to follow the same pathway like PMA, i.e., poly ADP-ribosylation and protein kinase C (PKC) pathway but to different extents.

2. Materials and methods

PMA, mezerein, phosphatidyl-L-serine, alcohol dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo, USA.

*Corresponding author (Fax, 091-11-6862663; Email.neeta@medinst.ernet.in).

2.1 *Cell culture*

Monolayer cultures of normal human diploid kidney cells (HDCS), mouse embryo fibroblast cells (NIH3T3) and transformed human epidermoid cells (A431) were grown in DMEM supplemented with 10% fetal calf serum and antibiotics. Cells were treated for varying periods of time with either PMA (25 ng/ml) or niezerein (125 ng/ml).

2.2 *Assay of poly ADP-ribose polymerase activity*

The cells were permeabilized and assayed for the enzyme activity (Jacobson *et al* 1980).

2.3 *Estimation of* NAD^+

NAD^+ was extracted with ice-cold 0·1 M NaOH containing 1mM niacinamide and estimated by the method of Jacobson *et al* (1979).

2.4 *Assay of acceptor proteins for poly ADP-ribose*

The acceptor proteins for poly ADP-ribose were isolated and characterized by the method of Adamietz and Rudolph (1984) as modified by Krupitza. and Cerutti (1989).

2.4a *Isolation of nuclei*: Nuclei were isolated from cells post tumour promoter treatment and released by homogenization, layered on top of a sucrose cushion, pelleted at 800 *g* in a refrigerated Sorvall centrifuge for 10 min.

2.4b *Extraction of histones*: Histones were extracted from the nuclear pellet, with 10 ml of 0·25 M H_2 SO_4 for 30 min, and once with 10 ml of 0·15 M H_2 SO for 15 min. Acid soluble proteins were precipitated by 25% TCA and centrifuged at 5500 *g* for 2 h at 4°C. The supernatant was discarded, the pellet was washed with cold ethanol and acetone and dissolved in 8 ml of buffer (6 a guanidine hydrochloride, 50 mM sodium phosphate, and 5 mM 2-mercaptoethanol, pH 6·5). The pH of the pellet solution containing the histones was adjusted to 8·2 by addition of 400 μl of 1 M morpholine buffer, pH 8·7. The poly (ADP-ribosylated) proteins were separated by boronate chromatography.

2.4c *Separation of poly ADP-ribosylated proteins*: PBA-30 column was activated as described by Krupitza and Cerutti (1989) before the application of nuclear protein. Unbound proteins were eluted with 6 M guanidine-HCl, 50 mM morpholine, pH 8·2 and poly ADP-ribosylated proteins were released with 6 M guanidine-HCL, 200 mM sodium phosphate buffer, pH 5·5. The eluate was dialyzed against 0·6% CH_3 COOH and 6M urea at 4°C; then lyophilized and dissolved in sample buffer (30 mM phosphoric acid, 1% SDS, l% B-mercaptoethanol, 4·5 a urea, pH 6·0). Histones were separated on 15% SDS-PAGE and the bands visualized by Coomasie blue staining. Low molecular weight proteins and histone-2A were used as standard marker.

2.5 *PKC assay*

PKC activity was determined by measuring Ca^{2+} and phospholipid dependent phosphorylation of lysine rich histones and determining the incorporation of [r $^{-32}$P] into histone from [r $^{-32}$P] ATP. The radioactivity incorporated was quantitated by scintillation counting (Castagna *et al* 1982) and PKC activity was expressed as pmol/min/mg protein.

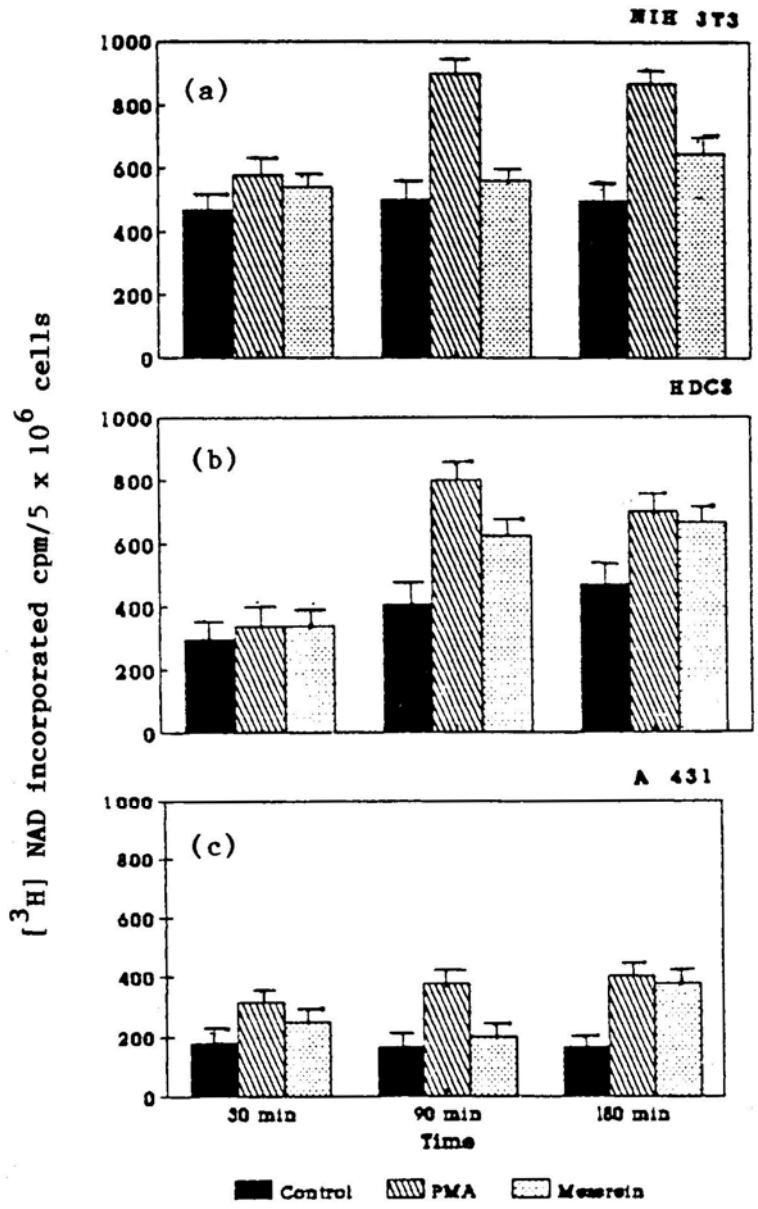

Figure 1. Effect of PMA and mezerein on PADPR polymerase activity. The cells were treated with PMA and mezerein for the indicated time periods and polymerase activity estimated. Each value is a mean ± SD of two separate experiments, each performed in duplicate.

3. Results

3.1 *Stimulation of poly ADP-ribosylation by tumour promoters*

A comparison of the effect of PMA and mezerein was made on poly ADPR poiymerase (PADPRP) activity and on the level of its substrate NAD^+. The basal level of the enzyme was higher in NIH 3T3 cells as compared to HDCS and A431 cells. PMA led to maximum stimulation of PADPRP activity by 73-76% in NIH 3T3 cells (figure 1a) and by 97% in HDCS cells (figure lb) at 90 min, as compared to the controls. In A431 cells PMA increased the enzyme activity by 80% as early as 30 min post treatment and the increase continued till 3 h (figure lc). In comparison mezerein showed a time dependent slow and steady increase in the enzyme activity in all the three cell types. The maximum stimulation observed in the enzyme activity at 180 min post treatment was by 34% in NIH 3T3, 2·2-fold in HDCS and 44% in A431 cells as compared to the controls. The rise in enzyme activity was accompanied by a drop in NAD^+ levels of 22% in NIH 3T3 (figure 2a), 30% in HDCS cells (figure 2b) at 90 min and by 44% in A431 cells at 3 h (figure 2c) post PMA treatment. Mezerein treatment showed a decrease of 16% in NIH 3T3. 32% in HDCS cells at 3 h, and of 34% in A431 cells at 90 min.

3.2 *Characterization of histones as poly ADP-ribose acceptors*

Histones H4, H2B, H1, A24 and H3d served as PADP-ribose acceptors in NIH 3T3 cells post PMA treatment, with H3d, A24 and H1 acting as major acceptors as noted from higher degree of intensity of the bands corresponding to these histones. Mezerein led to poly ADP-ribosylation of histones H1, H2B and A24, with H1 and H2B acting as major acceptors (figure 3a). In HDCS cells histones H3d, A24, H1, H2B, H3 and H4 appeared to be the acceptors post PMA treatment, with H3d, H2B and H1 as major acceptor whereas mezerein showed H1, H2B and H4 as the acceptors with H1 and H2B being the major acceptors (figure 3b).

3.3 *Tumour promoters led to PKC activation and translocation*

To address the question whether mezerein follows a similar mechanism of action as PMA, time dependent effects of PaA and mezerein were investigated on PKC activity in subcellular fractions i.e., cytosol and membrane. In NIH 3T3 and HDCS cells the basal PKC activity was found essentially in the cytosol fraction and was about 5-fold higher as compared to the membrane fraction (table 1). But in A431 cells the basal activity of PKC was more in the membrane fraction (table 1). Both PMA and mezerein caused a translocation of PKC from cytosol to membrane leading to a 14-fold increase in PKC activity in the membrane fraction at 15 min in NIH 3T3 cells, which decreased to 2·5-fold at 90 min post treatment. Similarly in HDCS cells the basal PKC activity was higher in cytosol. Treatment with PMA and mezerein resulted in increase in translocation of PKC to membrane by 7-fold and 3·6-fold respectively at 15 min. In A431 cells the membrane fraction showed higher basal PKC activity. Both PMA and mezerein further increased the membrane PKC activity by translocation from cytosol to the membrane at 15 min post treatment. This effect however decreased with time. From the results it appears that PMA and mezerein are mediating their tumour promoting effect at least partially through PKC activation.

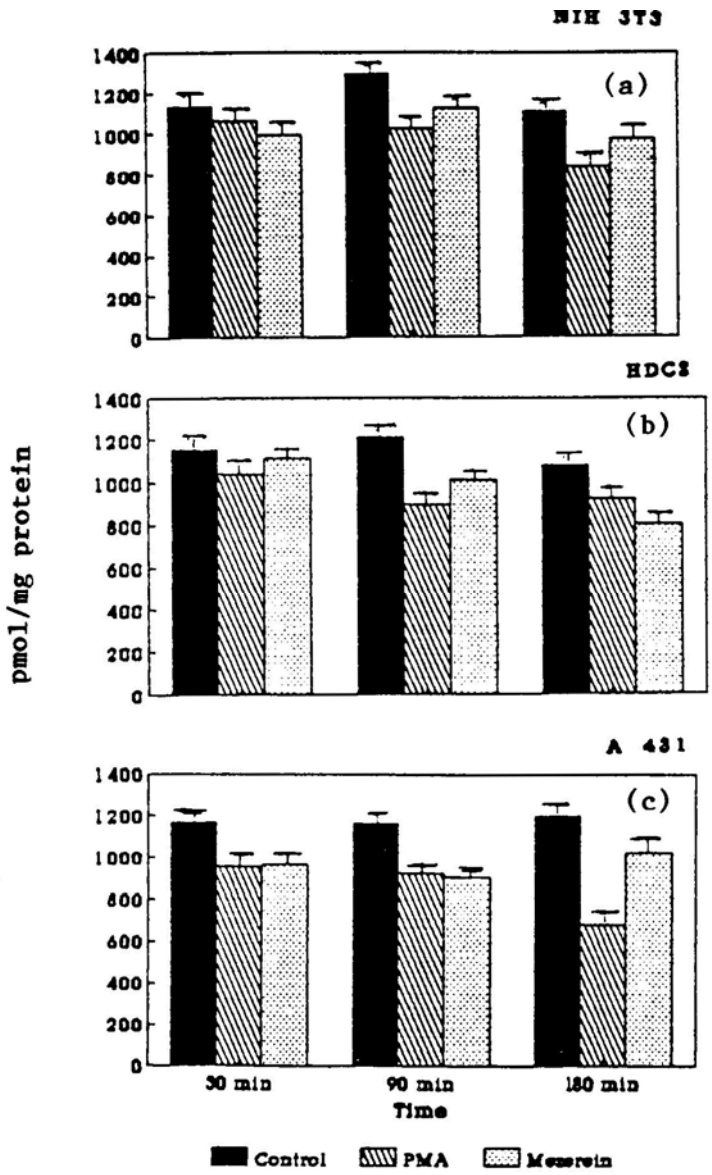

Figure 2. Effect of PMA and mezerein on NAD^+ levels. NAD^+ levels were determined post treatment with PMA and mezerein for the indicated time periods. Each value is a mean ± SD of two seperate experiments each performed in duplicate.

4. Discussion

Carcinogenesis is a multistage process where environmental exposure to physical, chemical and biological agents can act as major etiological factors. Poly ADP-ribosylation is a novel post translational modification of chromatin proteins catalyzed by the enzyme PADPRP using NAD^+ as substrate as a consequence of DNA strand

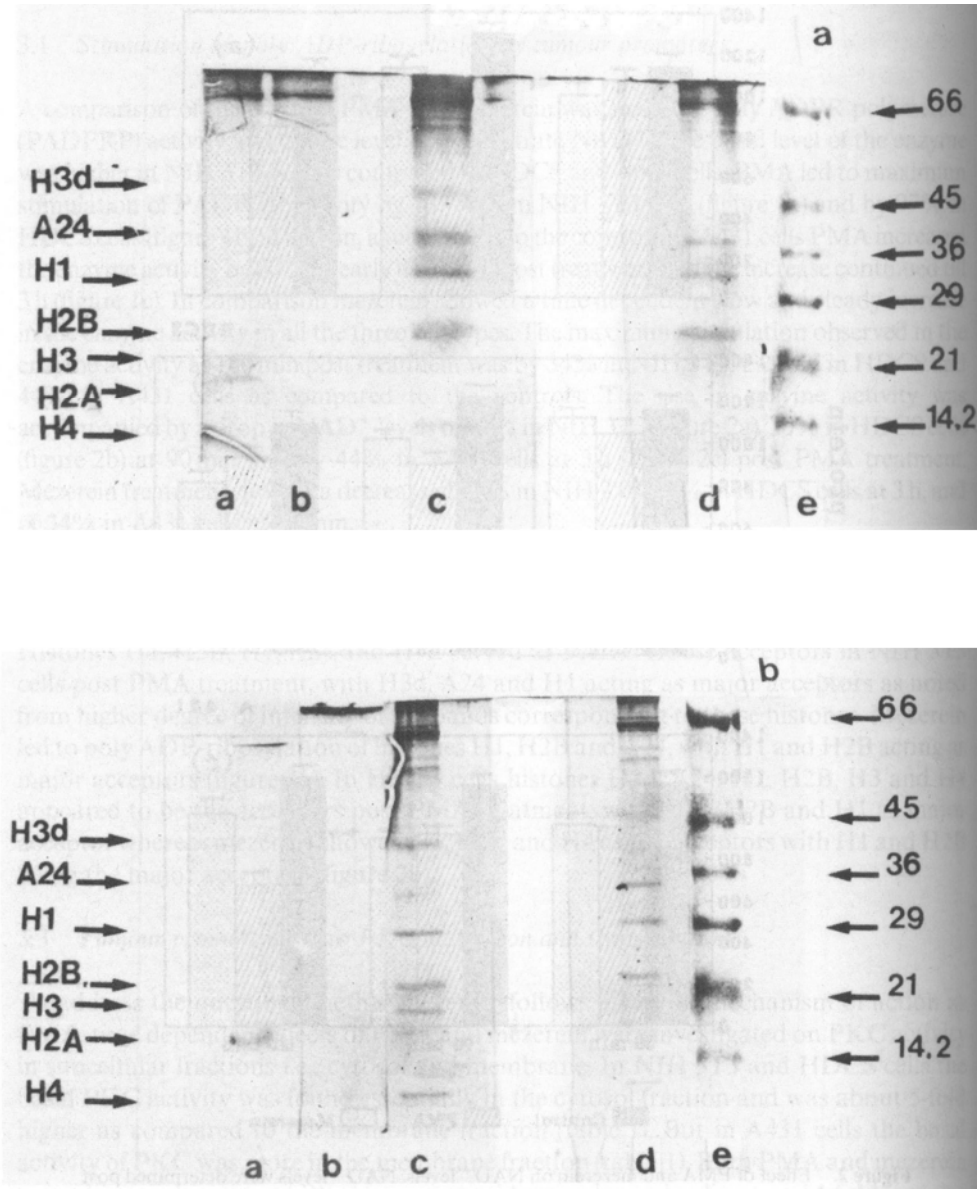

Figure 3. Poly ADP-ribosylation of histones proteins post PMA and mezerein treatment. NIH 3T3 (**a**) and HDCS (**b**) cells were treated with PMA and mezerein. The histones were isolated and histone acceptors of poly ADP-ribose analysed and characterized by SDS-PAGE as described in § 2. The lanes are (a) histone H2A, (b) control, (c) PMA, (d) mezerein and (e) Mol.wt marker.

breaks. Poly ADP-ribosylation has been reported to be involved in DNA replication, DNA repair, gene expression, cellular differentiation as well as in chromatin structure and plays a role in carcinogenesis (Boulikas 1991). PMA being a more potent tumour promoter showed an early and higher degree of stimulation of PADPRP activity in

Table 1. Time dependent effects of intracellular translocation of protein kinase C, post PMA and mezerein treatment. PKC activity was determined in the cytosol and membrane fractions at the indicated time points post tumour promoter treatment.

		PKC activity (pmol/min/mg protein)					
		NIH 3T3		HDCS		A431	
Treatment	Fraction	15 min	90 min	15 min	90 min	15 min	90 min
Control	Particulate	55 ± 9	51 ± 4	60 ± 7	56 ± 6	110 ± 12	120 ± 15
	Soluble	300 ± 27	286 ± 26	220 ± 21	195 ± 21	65 ± 7	60 ± 6
PMA	Particulate	281 ± 28	77 ± 6	280 ± 23	89 ± 6	150 ± 16	91 ± 10
	Soluble	20 ± 4	31 ± 4	40 ± 5	51 ± 5	30 ± 5	16 ± 3
Mezerein	Particulate	282 ± 29	71 ± 11	210 ± 19	84 ± 10	155 ± 17	84 ± 9
	Soluble	23 ± 5	36 ± 6	58 ± 8	61 ± 9	28 ± 3	16 ± 3

Each value is a mean ± SD of two separate experiments.

NIH 3T3 and HDCS cells, but a delayed stimulation in the transformed A431 cells. Mezerein being a weak tumour promoter led to a delayed and lower level of stimulation of the enzyme activity in all the three cell types. Thus it appears that the stimulation of PADPRP activity correlates with the extent of DNA damage, which in turn depends on the potency of the tumour promoter in question. The stimulation of enzyme activity by these two tumour promoters was transient, followed by a drop in enzyme activity but to different extents in the three cell types studied. The decrease in enzyme activity could be due to the inactivation of the enzyme. It has been reported that this nuclear enzyme besides catalysing the synthesis of the polymer can also act as a self acceptor for the polymer, poly ADP-ribose and thus undergo automodification which decreases the activity of the enzyme and may in turn lead to its inactivation (Singh *et al* 1985a, b). Our results are in agreement with previous reports where early stimulation of PADPRP activity by PMA has been shown in mouse embryo fibroblasts C3H10T1/2 and in human fibroblasts 3229 (Singh and Cerutti 1985; Lautier *et al* 1990; Singh *et al* 1985a, b; Singh 1990). NAD^+ serves as a substrate for the synthesis of the polymer, poly ADP-ribose. Thus with the polymer formation, the NAD^+ levels drop. Mezerein showed comparatively lower but more consistent enzyme stimulation and a steady drop in NAD^+ level. Poly ADPR synthesis has been correlated with DNA damage on the basis of several lines of evidence (Boulikas 1991). In the absence of DNA strand breaks histones are mono (ADP-ribosylated); however, when DNA strand breaks are introduced, histones appear as poly (ADP-ribosylated) species (Panzeter *et al* 1992). Pretreatment of mouse embryo fibroblasts with PMA results in endogenous poly ADP-ribosylation of histones (A24, H2B and H3) and the enzyme, PADPRP (Singh *et al* 1985a, b). In our study we found that although PMA and mezerein treatment resulted in poly ADP-ribosylation of several core and linker histones in both NIH 3T3 and HDCS cells, histones H1 and H2B were the common acceptors for mezerein irrespective of the cell type. Histone H1 has also been described to be the major histone acceptor in trout testis nuclei and in pancreatic nuclei and nucleosomes (Poirier *et al* 1982). Both H1 and H2B have been shown to serve as the major histone acceptor protein of PADP-ribose (De Murcia *et al* 1988). Histone H4 appeared to be a major and common acceptor for PMA induced Poly ADP ribosylation in both NIH 3T3 and HDCS cells but only in HDCS cells with mezerein. H4 and H3 are anchored more

tightly in the core structure in the nucleosomes than H2A and H2B. PMA appeared to share some common histone acceptors with mezerein. This could be because of some structural similarities it shares with mezerein and/or the similarity in its mode of action. The pattern of PKC activation by the potent tumour promoter PMA and the weak tumour promoter mezerein was very similar, and may be an early event in mediating some of their effects. Quantitation of the amount of PKC activity showed that both PMA and mezerein treatment led to a decrease in the cytosolic enzyme activity which could be accounted for by its simultaneous increase in the membrane fraction due to translocation. There are several reports showing the translocation of PKC from cytosol to the membrane fraction on PMA treatment (Nishizuka 1992; Da Silva *et al* 1990; Anderson *et al* 1985). Down regulation or depletion of soluble PKC probably is a common adaptive reaction of many cell types to PMA irrespective of its differentiating effect (Nishizuka 1984). Transformed cells may experience a chronic stimulation of PKC activity accompanied by down regulation of the enzyme like in colon carcinogenesis (Guillem *et al* 1987) and in transformed cells (Wolfmann *et al* 1987). Tumour promoters PMA and mezerein are known to increase intracellular free calcium (Miyake *et al* 1984) which in turn perhaps could induce PKC activity. Alternatively PKC translocation by PMA and mezerein may be an early event which may alter the availability of endogenous substrate and result in their biological response. Although mezerein does not have diacylglycerol like structure it is possible that it may cause analogous changes in plasma membrane by modifying the phospholipid bilayer structure. PKC may play a crucial role in signal transduction as it may be located at the crossroads of various pathways involving calcium, phosphatidyl inositol as well as tumour promoters. PKC has broad specificity and will phosphorylate a variety of substrates including histones (Shoji 1987). Phosphorylalion of poly ADPRP by PKC *in vitro* is shown (Tanaka 1987). An important role of PKC translocation upon activation may be the conveyance of signal through the cytoplasm to the nucleus by probably reorganization of the cytosol matrix. The results suggest that both phosphorylation by PKC and poly ADP-ribosylation may be key players in the alteration of chromatin structure and gene expression in tumour promotion by mezerein. The study suggests that there are unifying and multiple set of control mechanisms to translate the varying signals to the nucleus.

Acknowledgement

This work was supported by the Indian Council of Medical Research, New Delhi.

References

Adamietz P and Rudolph A 1984 ADP-ribosylation of nuclear proteins in vivo*; J. Biol. Chem.* **259** 986-993

Anderson W B, Estival A, Tapiovaara H and Gopalakrishna R 1985 Altered subcellular distribution of protein kinase C. Possible role in tumour promotion and the regulation of cell growth; *Adv. Cyclic. Nucl. Protein Phosphoryl. Res.* **19** 287-306

Birnboim H C 1986 DNA strand breaks in human leukocytes induced by superoxide anion, hydrogen peroxide and tumour promoters are repaired slowly compared to breaks induced by ionizing radiation; *Carcinogenesis* **7** 1511-1517

Boulikas T 1991 Relation between carcinogenesis, chromatin structure and poly (ADP-ribosylation); *Anticancer Res.* **11** 489-527

Castagna M, Takai Y, Kabibuchi K, Sano K, Kikkawa V and Nishizuka Y 1982 Direct activation of calcium activated, phospholipid dependent protein kinase by tumour promoting phorbol esters; *J. Biol. Chem*. **257** 7847-7851

Cerutti P 1985 Prooxidant states and tumour promotion; *Science* **227** 375-381

Da Silva C, Fan X Martelly I and Castagna M 1990 Phorbol esters mediate phospholipid free activation of rat brain protein kinase C; *Cancer Res*. **50** 2081-2087

De Murcia G, Huletsky A and Poirier G G 1988 Modulation of chromatin structure by poly ADP-ribosylation; *Biochem. Cell. Biol*. **66** 626-635

DiGiovanni 1992 Multistage carcinogenesis in mouse skin; *Pharmacol. Ther.* **54** 63-128

Fibac E, Agranomt I and Rachmilewitz E A 1984 Differentiation of human myeloid leukemia cells by phorbol esters: Correlation with tumour promotion; *Int. J. Cancer* **34** 451-457

Guillem J G, O'Brien C A, Fitzer C J, Forde K A, Logerfo P, Treat M and Weinstein I B 1987 Altered levels of protein kinase C and calcium dependent protein kinase in human colon carcinomas; *Cancer. Res*. **47** 2036-2039

Jacobson E L, Lange R A and Jacobson M K 1979 Pyridine nucleotide synthesis in 3T3 cells; *J. Cell. Physiol*. **99** 417-426

Jacobson M K, Levi V, Juarez-Salinas H, Burton R A and Jacobson E L 1980 Effect of carcinogenic N-alkyl N-nitro compounds on nicotinamide adenine dinucleotide metabolism; *Cancer Res*. **40** 1797-1802

Krupitza G and Cerutti P 1989 ADP-ribosylation of ADPR Transferase and topoisomerase I in intact mouse epidermal cells JB6; Biochemistry **28** 2034-2040

Lautier D, Poirier D, Boudreau A, Alaovi Jamai M A, Castonguary A and Poirier G 1990 Stimulation of Poly ADP-ribose synthesis by free radicals in C3H10T1/2 cells; Relationship with NAD metabolism and DNA breakage; *Biochem. Cell. Biol*. **68** 602-608

Miyake R A, Tanaka Y, Tsuda T, Kaibuchi K, Kikkawa V and Nishizuka Y 1984 Activation of PKC by non phorbol tumour promoter mezerein; Biochem. Biophys. Res. Commun. **121** 649-656

Mufron A, Fister S M, Verma A K, Gleason G L, Slaga T J and Boutwell R K 1979 Effect of 12-0 Tetradecanoyl-phorbol-13-Acetate and mezerein on epidermal ornithine decarboxylase activity iso-proterenol stimulated levels of cyclic 3-5 monophosphate and induction of mouse skin tumors in vivo; *Cancer Res*. **39** 4791-4795

Nishizuka Y 1984 The role of protein kinase C in cell surface signal transduction and tumour promotion; *Nature* (*London*) **308** 693-698

Nishizuka Y 1992 Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C; *Science* **258** 607-614

Panzeter P L, Realini C A and Althaus F R 1992 Noncovalent interactions of Poly ADP-ribose with histones; *Biochemistry* **31** 1379-1385

Poirier G G, De Murcia G, Jongstra-Bilen J, Niedergang C and Mandel P 1982 Poly ADP-ribosylation of polynucleosomes causes relaxation of chromatin structure; *Proc. Natl. Acad. Sci. USA* **79** 13423-13427

Shoji M, Girard P R, Charp P A, Koehler H P, Vogler W R and Kuo J F 1987 Effect of phorbol ester on translocation and down regulation of protein kinase C and phosphorylation of endogenous proteins in human acute myeloid leukemia cell line KG-1 and its phorbol ester-resistant subline KG-la; *Cancer Res*. **47** 6363-6370

Singh N and Cerutti P 1985 Poly ADP-ribosylation of histones in tumour promoter phorbol-12-myrestate-13-acetate treated mouse embryo fibroblasts C3H 10 T112 cells; *Biochem. Biophysics. Res. Commun.* **132** 811-819

Singh N, Leduc Y, Poirier G and Cerutti P 1985a Non histone chromosomal protein acceptors for poly ADP-ribose in PMA treated mouse embryo fibroblasts C3H 10T 1/2; *Carcinogenesis* **6** 1489-1493

Singh N, Poirier G and Cerutti P 1985b Tumor promoter phorbol-12-myristate-13-acetate induces poly (ADP) ribosylation in fibroblasts; *EMBO J*. **4** 1491-1494

Singh N 1990 Effect of tumour promoters on Poly ADP ribosylation in human epidermoid carcinoma HeP2 cells; *Int. J. Exp. Pathol.* **71** 809-814

Sun Y 1990 Free radicals, antioxidant enzymes and carcinogenesis; *Free Radical Biol. Med*. **8** 583-599

Tanaka Y, Koide S, Yoshihara K and Kanaiya K 1987 Poly (ADP-Ribose) synthetase is phosphorylated by protein kinase C in vitro*; Biochem. Biophys. Res. Commun*. **148** 709-717

Wolfman A, Wingrove T G, Blackshear P J and Macara I G 1987 Down-regulation of Protein Kinase C and of Endogenous 80 kDa substrate in transformed fibroblasts; *J. Biol. Chem*. **262** 16546-16552

Corresponding editor: M S SHALIA