

# Over-expression of protein kinase C- $\alpha$ enhances platelet-derived growth factor- and phorbol ester- but not calcium ionophore-induced formation of prostaglandins in NIH 3T3 fibroblasts

Günter Finkenzeller<sup>a</sup>, Frank Totzke<sup>b</sup>, Edith Fitzke<sup>b</sup>, Dieter Marmé<sup>b</sup> and Peter Dieter<sup>b,c</sup>

<sup>a</sup>Medizinische Universitätsklinik, Hugstetter Strasse 55, <sup>b</sup>Institut für Molekulare Zellbiologie, Mooswaldallee 1-9 and

<sup>c</sup>Biochemisches Institut, Hermann-Herder-Strasse 7, Universität Freiburg, D-7800 Freiburg, Germany

Received 28 January 1993; revised version received 5 March 1993

Over-expression of human protein kinase C- $\alpha$  in murine NIH 3T3 fibroblasts is associated with an increased platelet-derived growth factor- and phorbol ester-mediated formation of prostaglandins, whereas the calcium ionophore-induced release of arachidonic acid metabolites is unaffected; however, the differences of arachidonic acid and prostaglandin formation are much more pronounced with platelet-derived growth factor than with phorbol ester. Platelet-derived growth factor induces an identical elevation of intracellular free calcium in control and protein kinase C- $\alpha$  over-expressing cells; the phorbol ester has no effect on intracellular free calcium in both cell lines. These results demonstrate that protein kinase C- $\alpha$  may couple to arachidonic acid cascade in NIH 3T3 fibroblasts.

Over-expression; Protein kinase C; Ca<sup>2+</sup> release; Prostaglandin; Arachidonic acid

## 1. INTRODUCTION

Protein kinase (PK) C has been shown to play a crucial role in many cellular signal transduction pathways [1]. Molecular cloning and biochemical analysis have revealed that PKC exists as a family of related enzymes, the Ca<sup>2+</sup>-dependent isoforms  $\alpha$ ,  $\beta$  and  $\gamma$ , and the Ca<sup>2+</sup>-independent isoforms  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$  and  $\theta$  [1,2]. However, the exact role of the various isoenzymes in different cellular reactions has not been clarified in detail.

One cellular reaction to be under the control of PKC is the arachidonic acid (AA) cascade [3]. Stimulation of this cascade leads to the formation and release of AA, prostaglandins (PG) and leukotrienes. A phospholipase A<sub>2</sub> is thought to be the key enzyme, and PKC and calcium are reported to mediate activation of this cascade [4]. The involvement of the various isoforms of PKC in this cascade is controversial. While in rat macrophages PKC- $\beta$  is reported to mediate activation of AA release [5], in rat mesangial cells [6] and canine kidney cells [7] PKC- $\alpha$  and - $\epsilon$ , respectively, are thought

to be involved in the activation of this cascade. These data have been obtained by determining the isoenzyme pattern of the cells and/or by differential down-regulation of the different isoforms of PKC. Here, we investigate by over-expression studies whether PKC- $\alpha$  is able to couple to AA cascade in NIH 3T3 fibroblasts.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Human recombinant platelet-derived growth factor (hPDGF) BB was from Bissendorf Biochemicals (Hannover, Germany), 12-myristate 13-acetate (PMA) from Pharmacia (Freiburg, Germany), the calcium ionophore A 23187 and Fura-2 acetoxymethyl ester (Fura-2/AM) from Calbiochem (Gießen, Freiburg). Leighton tubes were purchased from Tecnomara (Fernwald, Germany). [5,6,8,9,11,12,14,15-<sup>3</sup>H]AA (150–230 Ci/mmol) was obtained from Amersham Buchler (Braunschweig, Germany). Cell culture media and sera were purchased from Gibco (Eggenstein, Germany). The antibodies against PGE<sub>2</sub> were a generous gift from Dr. Mollenhauer (Erlangen, Germany).

### 2.2. Cell culture

NIH 3T3 fibroblasts were stably transfected with the eukaryotic expression vector, pMxSVneo, containing hPKC- $\alpha$  (hPKC- $\alpha$  over-expressing cells), or the vector lacking any insert (control cells), respectively [8]. Cells were grown in Dulbecco's modified essential medium supplemented with 10% bovine calf serum. 48 h prior to the experiments the serum concentration was reduced to 1% to induce quiescence [8].

### 2.3. Determination of released [<sup>3</sup>H]AA and its metabolites

The cells were plated at a density of 250 000 cells/dish in 60 mm dishes containing 0.8  $\mu$ Ci/ml [<sup>3</sup>H]AA and incubated for 24 h. Then the medium was changed to the respective media containing 1% serum and

Correspondence address: P. Dieter, Institut für Molekulare Zellbiologie, Mooswaldallee 1-9, 7800 Freiburg, Germany. Fax: (49) (761) 518-3086.

Abbreviations: PKC, protein kinase C; AA, arachidonic acid; PG, prostaglandin; hPDGF, human recombinant platelet-derived growth factor; Fura-2/AM, Fura-2 acetoxymethyl ester; HPLC, high-performance liquid chromatography; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular concentration of free Ca<sup>2+</sup>; PMA, phorbol 12-myristate 13-acetate.

0.8  $\mu\text{Ci}$  [ $^3\text{H}$ ]AA. After 48 h the quiescent cells were washed thoroughly and incubated in Hanks solution without or with stimulus. After 60 min the media were removed, centrifuged and the supernatants analysed for released  $^3\text{H}$  label (AA and AA metabolites), or extracted for analysis by high-performance liquid chromatography (HPLC) [9].

#### 2.4. Determination of released $\text{PGE}_2$

Quiescent cells were incubated in Hanks solution without or with stimulus for 60 min and the amount of  $\text{PGE}_2$  in cell media was determined by enzyme-linked immunosorbent assay [10].

#### 2.5. Measurement of free intracellular $\text{Ca}^{2+}$ ( $[\text{Ca}^{2+}]_i$ )

Quiescent cells, attached to Leighton tubes, were loaded with 20  $\mu\text{M}$  Fura-2/AM for 60 min. Thereafter, the cells were washed thoroughly and the slides fixed with a special holder at a  $45^\circ$  angle in a thermostated cuvette containing 3 ml Hanks solution. Fluorescence measurements were performed with stirring at  $30^\circ\text{C}$  in a RF-5000 Shimadzu spectrofluorometer. The wavelength for excitation was  $335 \pm 5$  nm and  $380 \pm 5$  nm, and for emission  $490 \pm 5$  nm.  $[\text{Ca}^{2+}]_i$  was calibrated from the ratio of fluorescence measurements as described previously [11].

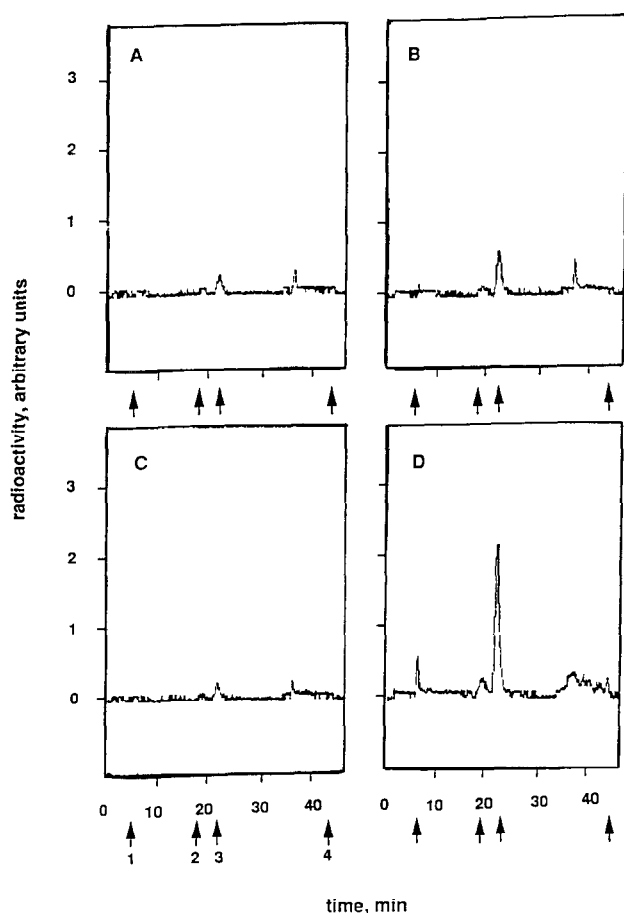


Fig. 1. HPLC profile of released  $^3\text{H}$ -labeled prostaglandins in control and hPKC- $\alpha$  over-expressing cells. Control (FT11 $\beta$ , upper panel) and hPKC- $\alpha$  over-expressing (N1A, lower panel) cells were grown as described in section 2.  $1 \mu\text{M}$  PMA (A,C) or  $50 \text{ ng/ml}$  PDGF (B,D) were added for 60 min, the cell supernatants removed and processed for analysis by HPLC. The position of the reference substances 6-keto  $\text{PGF}_{1\alpha}$  (1),  $\text{PGF}_{2\alpha}$  (2),  $\text{PGE}_2$  (3) and AA (4) are indicated by arrows. The peaks eluting between 34 and 42 min co-elute with hydroxyperoxytetraenoic acids. A typical set of data, reproduced three times, is given.

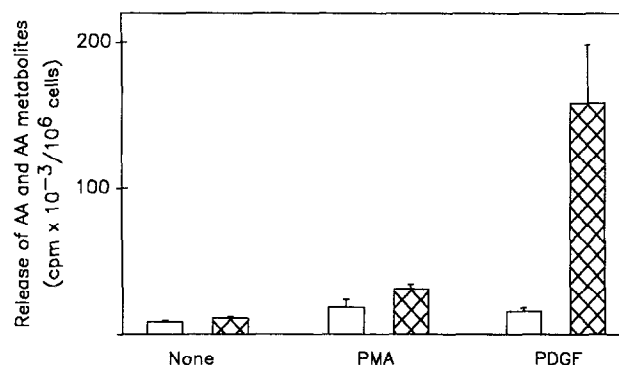


Fig. 2. Release of AA metabolites of control and hPKC- $\alpha$  over-expressing cells. Control (FT11 $\beta$ , upper panel) and hPKC- $\alpha$  over-expressing (N1A, lower panel) cells were grown as described in section 2.  $1 \mu\text{M}$  PMA or  $50 \text{ ng/ml}$  PDGF were added for 60 min, the cell media centrifuged and the amount of  $^3\text{H}$  label in the supernatants determined. Results are means  $\pm$  S.D. of three independent experiments.  $P$  values were calculated using Student's  $t$ -test analysis (control cells vs hPKC- $\alpha$  over-expressing cells): none, n.s.; PMA,  $P < 0.02$ ; PDGF,  $P < 0.004$ ; n.s., not significant.

### 3. RESULTS

Over-expression of hPKC- $\alpha$  in NIH 3T3 fibroblasts has been shown to result in increased hPKC- $\alpha$  mRNA, hPKC- $\alpha$  protein and PKC activity [11]. Here we have investigated the effect of hPKC- $\alpha$  over-expression on the AA cascade in these cells. In order to stimulate PKC, we used the phorbol ester, PMA, and PDGF (the latter is known to activate PKC via the endogenous production of diacylglycerol [12]). In control cells PMA and PDGF lead to the formation of mainly  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  (Fig. 1A and B). In hPKC- $\alpha$  over-expressing cells, PMA induces a similar formation and release of prostaglandins (Fig. 1C), while upon addition of PDGF an increased formation of  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$  and 6-keto  $\text{PGF}_{1\alpha}$  is measured (Fig. 1D). Similar results were obtained by measuring  $^3\text{H}$  label released into the cell media from [ $^3\text{H}$ ]AA-prelabeled cells (Fig. 2). In the media of unstimulated control and hPKC- $\alpha$  over-expressing cells only small amounts of radioactivity could be determined; PMA leads to a significant increase of  $^3\text{H}$  label released from both cell lines which was slightly higher in hPKC- $\alpha$  over-expressing cells (Fig. 2). In control cells PDGF induces a release of radioactivity comparable to PMA, whereas in hPKC- $\alpha$  over-expressing cells the PDGF-induced release of  $^3\text{H}$  label is enhanced by a factor of about 10 (Fig. 2).

Similar results were obtained when the amounts of  $\text{PGE}_2$  in cell media were determined immunologically (Fig. 3). Unstimulated cells show small amounts of  $\text{PGE}_2$  in the media. Addition of PMA leads to an increased  $\text{PGE}_2$  release in both cell lines which was higher in hPKC- $\alpha$  over-expressing cells than in control cells. PDGF shows a similar formation of  $\text{PGE}_2$  as PMA in control cells and a 3–4-fold enhanced  $\text{PGE}_2$  release in

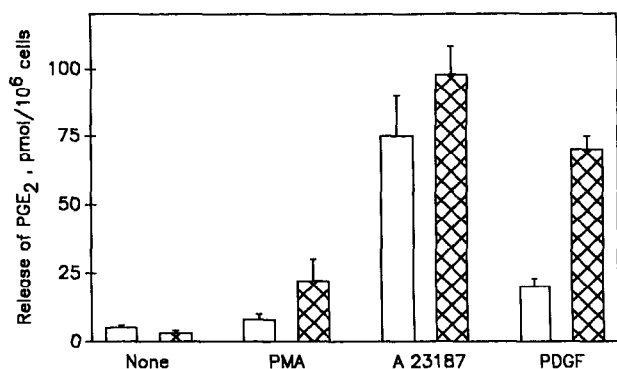


Fig. 3. Release of PGE<sub>2</sub> from control and hPKC- $\alpha$  over-expressing cells. Control (FT11 $\beta$ , upper panel) and hPKC- $\alpha$  over-expressing (N1A, lower panel) cells were grown as described in section 2. 1  $\mu$ M PMA, 50 ng/ml PDGF or 10  $\mu$ M A 23187 were added for 60 min, the cell media centrifuged and the amount of PGE<sub>2</sub> in the supernatants determined by enzyme-linked immunosorbent assay. Results are means  $\pm$  S.D. of three independent experiments. *P* values were calculated using Student's *t*-test analysis (control cells vs. hPKC- $\alpha$  over-expressing cells): none, n.s.; PMA, *P* < 0.04; A 23187, n.s.; PDGF, *P* < 0.0001; n.s., not significant.

hPKC- $\alpha$  over-expressing cells (Fig. 3). The calcium ionophore A 23187 induces in both cell lines a formation of PGE<sub>2</sub> comparable to the PDGF-induced PGE<sub>2</sub>-release in hPKC- $\alpha$  over-expressing cells (Fig. 3).

Addition of PDGF to NIH 3T3 fibroblasts has been shown recently to induce the formation of inositol phosphates and a biphasic change of [Ca<sup>2+</sup>]<sub>i</sub> [11,13]. After about 1–2 min a maximum [Ca<sup>2+</sup>]<sub>i</sub> was obtained which then declined to a slightly elevated sustained level when compared to basal [Ca<sup>2+</sup>]<sub>i</sub> [11]. Since it is postulated that the AA cascade is also controlled by calcium ions [14], [Ca<sup>2+</sup>]<sub>i</sub> was measured in control and hPKC- $\alpha$  over-expressing cells. Fig. 4 shows typical [Ca<sup>2+</sup>]<sub>i</sub> responses induced by PDGF in control (A) and hPKC- $\alpha$  over-expressing (B) cells. Both cell lines show a latency period (2–3 min) with no significant change in basal [Ca<sup>2+</sup>]<sub>i</sub>, followed by an increase in [Ca<sup>2+</sup>]<sub>i</sub>, which subse-

quently declined towards a slightly elevated sustained level (Fig. 4). The basal and peak values of [Ca<sup>2+</sup>]<sub>i</sub> are almost identical in both cell lines (Table I). Addition of PMA has no effect on [Ca<sup>2+</sup>]<sub>i</sub> in control and hPKC- $\alpha$  over-expressing cells (Table I).

#### 4. DISCUSSION

In the present study we could demonstrate that over-expression of hPKC- $\alpha$  enhances PDGF- and PMA-mediated release of prostaglandins in NIH 3T3 fibroblasts whereas the A 23187-induced release of prostaglandins was unaffected by over-expression of hPKC- $\alpha$ . However, the differences of the release of AA and PGE<sub>2</sub> was more pronounced with PDGF than with PMA. Furthermore, the PDGF-induced elevation of [Ca<sup>2+</sup>]<sub>i</sub> was not influenced by hPKC- $\alpha$ ; PMA has no effect on [Ca<sup>2+</sup>]<sub>i</sub> in control and hPKC- $\alpha$  over-expressing cells.

These results demonstrate that hPKC- $\alpha$  couples to AA cascade in NIH 3T3 cells but that stimulation of hPKC- $\alpha$  alone (with phorbol ester) without a concomitant elevation of [Ca<sup>2+</sup>]<sub>i</sub> (with PDGF) is insufficient to activate maximally the release of AA and synthesis of prostaglandins in these cells. PKC- $\alpha$  has been shown recently to mediate release of AA in canine kidney cells

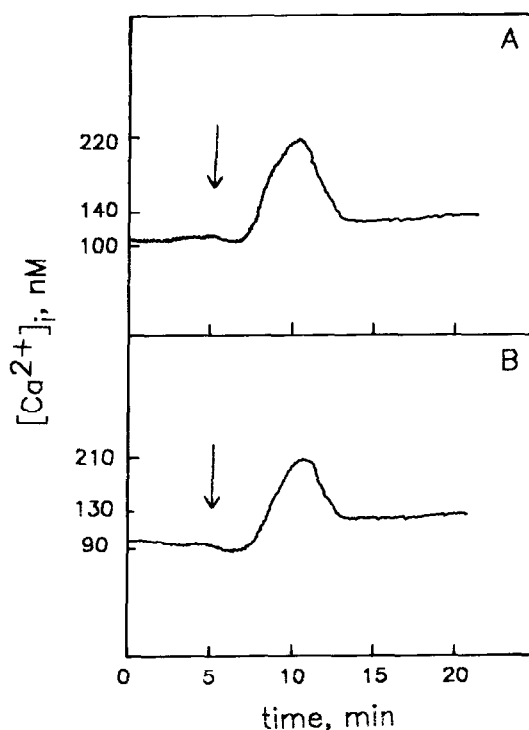


Fig. 4. [Ca<sup>2+</sup>]<sub>i</sub> responses to PDGF in control and hPKC- $\alpha$  over-expressing cells. Measurements of [Ca<sup>2+</sup>]<sub>i</sub> in monolayers of control (FT11 $\beta$ ) and hPKC- $\alpha$  over-expressing (N1A) cells were carried out as described in section 2. PDGF (50 ng/ml) was added as indicated by the arrows to control (A) and hPKC- $\alpha$  over-expressing (B) cells about 15 min after the slides were placed into the cuvettes.

Table I  
Effect of PMA and PDGF on [Ca<sup>2+</sup>]<sub>i</sub> in control and hPKC over-expressing cells

Addition	Maximal [Ca <sup>2+</sup> ] <sub>i</sub> (nM)	
	Control cells	hPKC- $\alpha$ over-expressing cells
None	98 $\pm$ 39	87 $\pm$ 29
PMA	89 $\pm$ 23	91 $\pm$ 15
PDGF	203 $\pm$ 33	207 $\pm$ 23

Measurements of [Ca<sup>2+</sup>]<sub>i</sub> in monolayers of control (FT11 $\beta$ ) and hPKC- $\alpha$  over-expressing (N1A) cells were carried out as described in Materials and Methods. PMA (1  $\mu$ M) or PDGF (50 ng/ml) were added 15 min after the slides were placed into the cuvettes. Values represent means  $\pm$  S.D. of 2–4 independent experiments.

[7], whereas in rat macrophages [5] and rat mesangial cells [6] PKC- $\beta$  and PKC- $\epsilon$  have been reported to be involved in activation of AA cascade, respectively. It seems therefore that different isoforms of PKC are able to trigger the release of AA and formation of prostaglandins. The data presented in this study indicate additionally that the effect of a distinct PKC isoform on cellular metabolism might be better demonstrated by using a physiological agent like PDGF (which induces the formation of the endogenous PKC activator diacylglycerol and a concomitant elevation of  $[Ca^{2+}]_i$ ) than by direct activation of PKC by phorbol ester.

*Acknowledgements.* This work was supported by grants from the Bundesministerium für Forschung und Technologie (Förderkennzeichen 01 GA 8816/0), Bonn/bad Godesberg.

## REFERENCES

- [1] Farago, A. and Nishizuka, Y. (1990) FEBS Lett. 268, 350–354.
- [2] Azzi, A., Boscoboinik, D. and Hensey, C. (1992) Eur. J. Biochem. 208, 547–557.
- [3] Axelrod, J. (1990) Bioch. Soc. Trans. 18, 503–507.
- [4] DeEnnis, E.A., Rhee, S.G., Billah, M.M. and Hannun, Y.A. (1991) FASEB J. 5, 2068–2077.
- [5] Duyster, J., Hidaka, H., Decker, K. and Dieter, P. (1992) Biochem. Biophys. Res. Commun. 183, 1247–1253.
- [6] Huwiler, A., Fabbro, D. and Pfeilschifter, J. (1991) Biochem. J. 279, 441–445.
- [7] Godson, C., Weiss, B.A. and Insel, P.A. (1990) J. Biol. Chem. 265, 8369–8372.
- [8] Finkenzeller, G., Marmé, D. and Hug, H. (1992) Cell Signal. 4, 163–177.
- [9] Dieter, P., Krause, H. and Schulze-Specking, A. (1990) Eicosanoids 3, 45–51.
- [10] Reinke, M., Piller, M. and Brune, K. (1989) Prostaglandins 18, 123–133.
- [11] Totzke, F., Hug, H., Fitzke, E., Marmé, D. and Dieter, P. (1992) FEBS Lett. 308, 125–129.
- [12] Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D. (1984) Biochem. J. 222, 195–201.
- [13] Totzke, F., Marmé, D. and Hug, H. (1992) Eur. J. Biochem. 203, 633–639.