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Over-expression of human phospholipase C-γ2 enhances platelet-derived growth factor-induced mobilization of intracellular Ca^{2+} and the release of arachidonic acid and prostaglandins in NIH 3T3 fibroblasts

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Over-expression of human phospholipase C-γ2 in murine NIH 3T3 fibroblasts has been shown to result in an increased platelet-derived growth factor-mediated formation of inositol phosphates. Here we show that phospholipase C-γ2 over-expression is associated with an increased platelet-derived growth factor-mediated release of arachidonic acid, prostaglandin E_2, 6-keto prostaglandin $F_{1\alpha}$ and prostaglandin $F_{2\alpha}$. The phorbol ester, calcium ionophore- and fluoride-induced release of arachidonate and its metabolites is not affected by phospholipase C-γ2 over-expression. Over-expression of phospholipase C-γ2 is also associated with an enhancement of platelet-derived growth factor-induced change in intracellular Ca^{2+}. These results demonstrate that stimulation of recombinant human phospholipase C-γ2 induces a change in the intracellular Ca^{2+} concentration, a release of arachidonic acid and formation of prostaglandins in NIH 3T3 cells. In control cells platelet-derived growth factor-induced activation of arachidonic acid cascade is rate-limited by the endogenous phospholipase C.

Phospholipase C-γ2; Over-expression; Arachidonic acid; Prostaglandin; Ca^{2+} release

1. INTRODUCTION

The human phospholipase C-γ2 (hPLC-γ2) cDNA has recently been cloned [1] and characterized by over-expression in NIH 3T3 fibroblasts [2]. The hPLC-γ2 was shown to be activated by platelet-derived growth factor BB (PDGF BB) [2]. Activation of phospholipase C results in the formation of second messengers, such as inositol 1,4,5-trisphosphate and diacylglycerol, which leads to intracellular Ca^{2+} mobilisation and activation of protein kinase C (PKC), respectively [3]. PKC and Ca^{2+} are important mediators in a number of intracellular reactions including stimulation of phospholipase A_2 [3–5]. Activation of phospholipase A_2 subsequently results in the liberation of arachidonic acid (AA) from membrane lipids and the formation of eicosanoids, important intercellular mediators in inflammation, cancer and other cellular processes [6–8]. To investigate the intracellular events leading to activation of the AA cascade we compared changes in the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the release of AA and formation of prostaglandins (PG) in control and in NIH 3T3 fibroblasts over-expressing hPLC-γ2.

Abbreviations: hPLC-γ2, human phospholipase C-γ2; PDGF BB, platelet-derived growth factor B-chain homodimer; PKC, protein kinase C; AA, arachidonic acid; PG, prostaglandin; PMA, phorbol 12-myristate 13-acetate; BSA, bovine serum albumin; MEM, minimum essential medium; HPLC, high-performance liquid chromatography; $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Human recombinant PDGF BB was from Bissendorf Biochemicals (Hannover, Germany), phorbol 12-myristate 13-acetate (PMA) was from Pharmacia (Freiburg, Germany) and the calcium ionophore, A 23187 was from Sigma (München, Germany). Fura-2 acetoxymethyl ester (Fura-2/AM) was obtained from Calbiochem (Giessen, Germany). Leighton tubes were from Tecnomara (Fernwald, Germany). [5,6,8,9,11,12,14,15-^{3}H]AA (150–230 Ci/mmol) was purchased from Amersham (Braunschweig, Germany). Purified bovine serum albumin (BSA) was from Behring AG (Marburg, Germany). Cell culture media were purchased from Gibco (Eggenstein, Germany). The antibodies against PGE_2 were a generous gift from Dr. Mollenhauer (Erlangen, Germany).

2.2. *Cell culture*

NIH 3T3 cells were transfected with the eucaryotic expression vector, pMxSVneo, containing hPLC-γ2, or the vector, pMxSVneo, lacking any insert (control cells), respectively [2]. Cells were grown in minimum essential medium (MEM)/10% fetal calf serum. For the AA release assays, cells were plated at a density of 30,000 cells/well in 24-well cluster plates in MEM/10% serum containing 0.8 μCi/ml [^{3}H]AA and incubated for 24 h. The medium was then changed to the respective media containing 1% serum and 0.8 μCi [^{3}H]AA for an additional 48 h to induce quiescence. For high performance liquid chromatography (HPLC) analysis, cells were plated at a density of 250,000 cells/dish in 60-mm dishes, as described above.

2.3. *Determination of released [^{3}H]AA and its metabolites*

Cells were plated and grown as described in the previous section.

Quiescent cells were washed thoroughly and incubated without or with stimulus in Hank's solution either with (determination of released [^{3}H]AA) or without (HPLC analysis of released [^{3}H]AA metabolites) 2% (w/v) BSA. After indicated times the media were removed, centrifuged and (i) the radioactivity determined in the supernatants by scintillation counting or (ii) the supernatants extracted for analysis of released label by HPLC [9]. In the presence of BSA, the radioactivity released into the medium consists predominantly of [^{3}H]AA ([9], data not shown). In experiments studying the effect of extracellular Ca^{2+} depletion on PDGF-stimulated AA release, the assay was carried out in Ca^{2+}-free Hank's/BSA solution.

2.4. *Determination of PGE_2*

Cells were incubated in Hank's solution without or with stimulus. After 60 min the media were removed and centrifuged. PGE_2 was determined in the supernatants by specific enzyme-linked immunosorbent assay [10].

2.5. *Measurements of $[Ca^{2+}]_i$*

Cells, attached to Leighton tube slides, were incubated in MEM/1% serum and 20 μM Fura-2/AM for 60 min at 37°C. Thereafter, the cells were washed thoroughly with Hank's solution and the slides were fixed with a special holder at a 45° angle in a thermostatted cuvette containing 3 ml Hank's solution. Fluorescence measurements were performed with stirring at 30°C in a RF-5000 Shimadzu spectrofluorometer. The wavelength for excitation was 335 ± 5 nm and 380 ± 5 nm and for emission 490 ± 5 nm. $[Ca^{2+}]_i$ was calibrated from the ratio of fluorescence measurements as described previously [11].

3. RESULTS

Over-expression of hPLC-γ2 in NIH 3T3 fibroblasts has been shown to result in an increase of hPLC-γ2 mRNA, hPLC-γ2 protein and an approximately 3-fold-enhanced PDGF BB-mediated formation of inositol phosphates [2]. Here we have investigated the effect of PDGF BB on the release of AA. While in control cells (transfected with the vector pMxSVneo only) and hPLC-γ2-over-expressing cells (transfected with pMxSVneo/hPLC-γ2) the basal release of AA was almost identical, the PDGF BB-induced AA release was significantly enhanced in hPLC-γ2 over-expressing cells (Fig. 1). The HPLC pattern of released AA and its metabolites from unstimulated and PDGF BB-stimulated cells is shown in Fig. 2. In the media of unstimulated cells (Fig. 2A,C) only small amounts of AA and AA metabolites were detected independently whether the cells over-expressed hPLC-γ2 (Fig. 2C) or not (Fig. 2A). Addition of PDGF BB led to the formation of mainly PGE_2, 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (Fig. 2B,D). hPLC-γ2 over-expressing cells showed an enhanced release of all prostaglandins (Fig. 2D). Integration of the peak areas (see legend of Fig. 2) reveals that the degree of stimulation is similar for all prostaglandins and AA, indicating an effect of hPLC-γ2 over-expression on phospholipase A_2 and not on cyclooxygenase or following enzymes in the AA cascade. A stimulatory effect of hPLC-γ2 over-expression on AA cascade was also observed when the levels of PGE_2 in cell media were determined immunologically in three different control and hPLC-γ2 over-expressing cell lines (Fig. 3). All hPLC-γ2 over-expressing cells responded to PDGF BB with an about 2–3-fold higher release of PGE_2, as compared to control cells. Unlike PDGF BB, phorbol ester or calcium ionophore induced a similar release of AA in

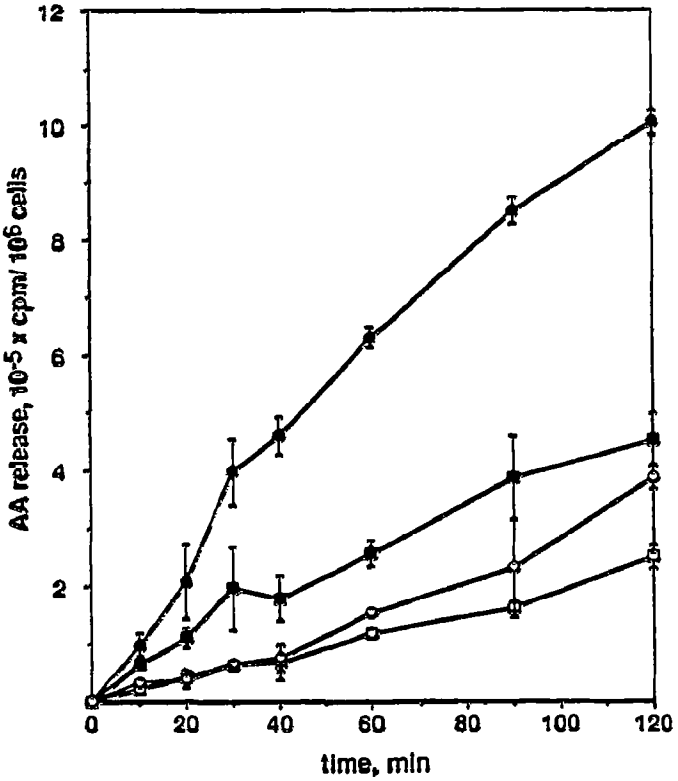

Fig. 1. Kinetics of the appearance of [^{3}H]AA in cell media from unstimulated and PDGF BB-stimulated cells. Control (clone 11β, squares) and hPLC-γ2-over-expressing (clone 13α, circles) cells were grown as described in section 2. When the cells were quiescent, the medium was replaced by Hank's solution with 2% (w/v) BSA containing no (open symbols) or 50 ng/ml PDGF BB (closed symbols). At the times indicated, the radioactivity in the cell supernatants was determined. Results are means ± S.D. of three independent experiments.

Table I

Effect of extracellular Ca^{2+} depletion on $[Ca^{2+}]_i$ parameters in control and hPLC-γ2 over-expressing cells

	$[Ca^{2+}]_i$ (nM)			
	Control cells		hPLC-γ2 over-expressing cells	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
Basal	59 ± 18	98 ± 39	60 ± 24	117 ± 44
Peak	87 ± 13	171 ± 24	259 ± 55	562 ± 132
Sustained	71 ± 22	125 ± 15	78 ± 37	206 ± 51

Measurements of $[Ca^{2+}]_i$ in monolayers of control (11β) and hPLC-γ2 over-expressing (13α) cells were carried out as described in the legend to Fig. 5. In experiments studying the effect of extracellular Ca^{2+} depletion, the cells were incubated for 15 min in Ca^{2+}-free Hank's solution prior to measurements. Values represent means ± S.D. of 6–9 independent experiments.

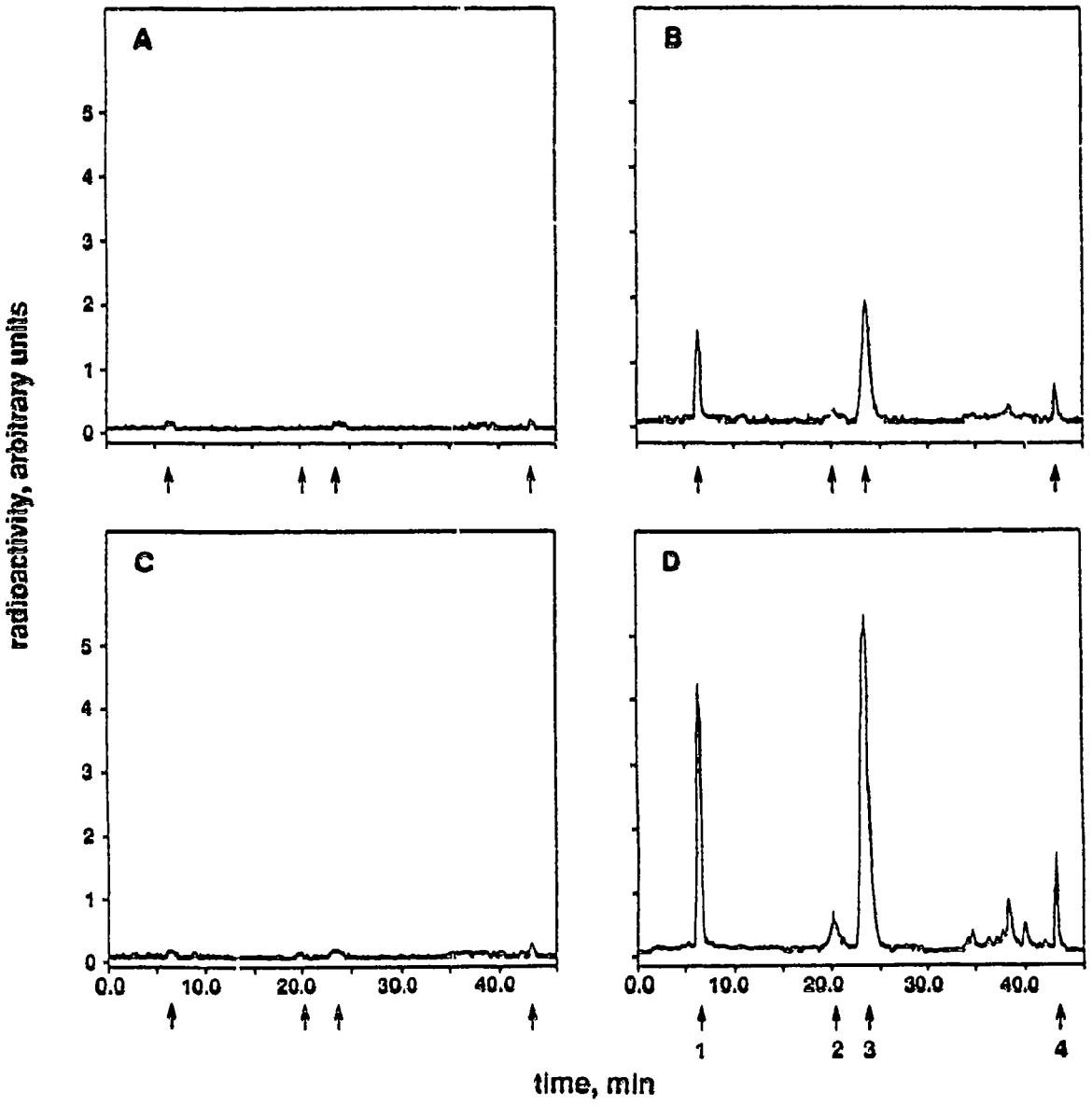

Fig. 2. HPLC profile of ^{3}H-labeled prostaglandins and arachidonate from control and hPLC-γ2 over-expressing cells. Control (11β, upper panels) and hPLC-γ2 over-expressing (13α, lower panels) cells were grown as described in section 2. When the cells were quiescent, media were replaced by Hank's solution containing no (A,C) or 50 ng/ml PDGF BB (B,D). After 60 min the supernatants were removed for processing and analysis by HPLC. The position of reference substances 6-keto $PGF_{1\alpha}$ (1), $PGF_{2\alpha}$ (2), PGE_2 (3) and AA (4) are indicated by arrows. The peaks eluting between 34 and 42 min co-elute with hydroxyeicosatetraenoic acids. Integration of the peaks gave the following results (dpm): control cells (+PDGF BB) – 6 keto $PGF_{1\alpha}$ (4,377), $PGF_{2\alpha}$ (1,512), PGE_2 (11,254), AA (1,377); hPLC-γ2 over-expressing cells (+PDGF BB) – 6 keto $PGF_{1\alpha}$ (14,658), $PGF_{2\alpha}$ (3,157), PGE_2 (39,048), AA (3,744). A typical set of data, reproduced three times, is given.

control and hPLC-γ2 over-expressing cells (Fig. 4), indicating that the mechanisms leading to AA release under maximal stimulation of PKC (with phorbol ester) or under high Ca^{2+} levels (with A 23187) have not been changed by the over-expression of hPLC-γ2. Agonists like bradykinin [2] or bombesin (data not shown), which are reported to activate PLC via G-proteins [12], only marginally induce a generation of inositol phosphates in control and hPLC-γ2 over-expressing NIH 3T3 cells. These agonists also exerted only a very small stimulatory effect on AA release in both cell lines (data not shown). Fluoride, a known activator of G-proteins and G-protein-linked PLC [12,13] induced a significant release of AA (Fig. 4) and inositol phosphates (data not shown). However, the amount of released AA (Fig. 4) and inositol phosphates was similar in control and hPLC-γ2 over-expressing cells.

Fig. 5 shows typical $[Ca^{2+}]_i$ responses induced by PDGF BB in control (Fig. 5B) and hPLC-γ2 over-expressing (Fig. 5A) cells. Both cell lines showed a sub-

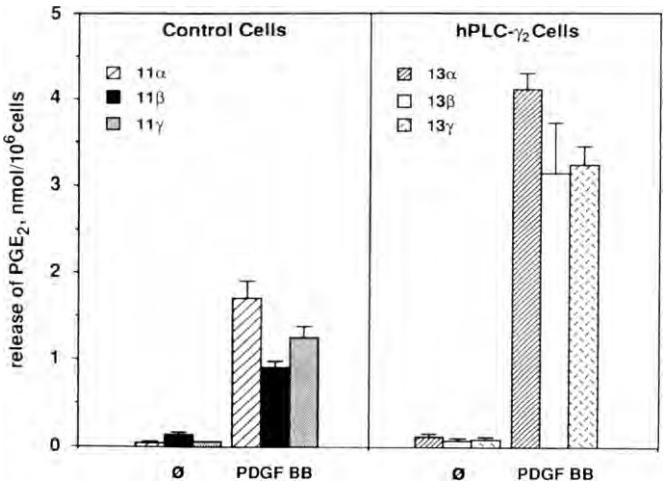

Fig. 3. Release of PGE_2 from control and hPLC-γ2 over-expressing cells. Control and hPLC-γ2 over-expressing cells were grown as described in section 2. Then the media were changed to Hank's solution containing no stimulus (Ø) or PDGF BB (50 ng/ml). After 60 min the amount of PGE_2 in supernatants was determined by enzyme-linked immunosorbent assay. Results are means ± S.D. of six independent experiments.

stantial but similar latency period (2–3 min) with no significant change in basal $[Ca^{2+}]_i$, followed by an increase in $[Ca^{2+}]_i$ which subsequently declined in both cell lines towards a slightly elevated sustained level. The basal values for $[Ca^{2+}]_i$ were almost identical in control and hPLC-γ2 over-expressing cells (Table I). Following PDGF BB stimulation, $[Ca^{2+}]_i$ increased in control cells about 2-fold, whereas in hPLC-γ2 over-expressing cells

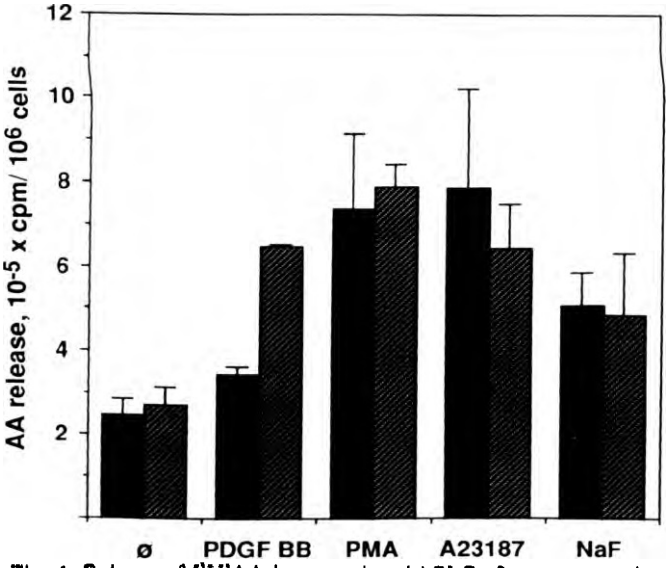

Fig. 4. Release of [^{3}H]AA in control and hPLC-γ2 over-expressing cells. Control (11β, filled bars) and hPLC-γ2 over-expressing (13α, hatched bars) cells were grown as described in section 2. The media were then replaced by Hank's solution with 2% (w/v) BSA containing no stimulus (Ø), PDGF BB (50 ng/ml), A 23187 (10 μM), PMA (1 μM) or NaF (30 mM NaF/50 μM $AlCl_3$). After 60 min the radioactivity in the supernatants was determined. Results are means ± S.D. of three independent experiments.

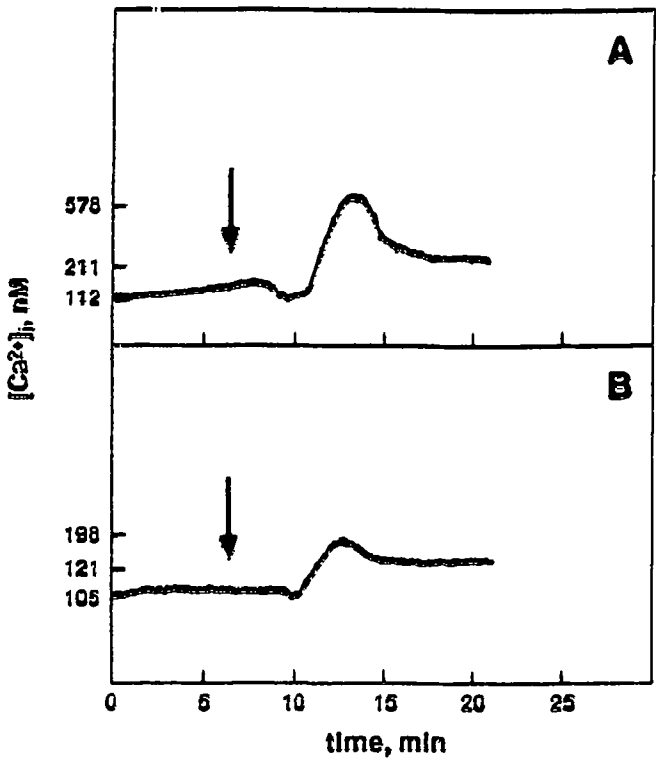

Fig. 5. $[Ca^{2+}]_i$ responses to PDGF BB in control and hPLC-γ2 over-expressing cells. Monolayers of cells, attached to Leighton tube slides, were loaded with Fura-2/AM and $[Ca^{2+}]_i$ was determined as described in section 2. PDGF BB (50 ng/ml) was added as indicated by the arrow to control (11β, panel B) and hPLC-γ2 over-expressing (13α, panel A) cells about 15 min after the slides were placed into the cuvette.

an about 5-fold increase in $[Ca^{2+}]_i$ was measured. 8–10 min after addition of PDGF BB, $[Ca^{2+}]_i$ declined to elevated sustained levels in both cell lines (Table I). In the absence of extracellular Ca^{2+}, PDGF BB was still able to initiate an increase of $[Ca^{2+}]_i$, however, the peak levels were smaller and $[Ca^{2+}]_i$ declined to levels compa-

Table II

Effect of extracellular Ca^{2+}-depletion on release of [^{3}H]AA in control and hPLC-γ2 over-expressing cells

	AA release (10^{-3} × cpm/10^6 cells)	
	Control cells	hPLC-γ2 over-expressing cells
−Ca		
−PDGF	201 ± 6	263 ± 21
+PDGF	193 ± 4	338 ± 25
+Ca		
−PDGF	311 ± 41	360 ± 53
+PDGF	390 ± 23	761 ± 28

Control (clone 11β) and hPLC-γ2 over-expressing (clone 13α) cells were grown as described in section 2. When the cells were quiescent, the medium was replaced by Hank's solution containing 2% (w/v) BSA without or with 1.3 mM $CaCl_2$. 60 min after addition of PDGF BB (50 ng/ml) the radioactivity in the supernatants was determined. Results are means ± S.D. of three independent experiments.

rable to basal $[Ca^{2+}]_i$. Nevertheless, the peak differences between control and hPLC-γ2 over-expressing cells were qualitatively similar in the absence and presence of Ca^{2+} (Table I). Under Ca^{2+}-free conditions, PDGF induced no (control cells) or only little (hPLC-γ2 over-expressing cells) AA release (Table I).

4. DISCUSSION

In the present study we could demonstrate that over-expression of hPLC-γ2 enhances PDGF BB-induced changes in $[Ca^{2+}]_i$ and PDGF BB-induced release of AA and prostaglandins. Together with the recent finding that hPLC-γ2 over-expression increases PDGF BB-induced generation of inositol phosphates [2] it may be concluded that the altered Ca^{2+} response in hPLC-γ2 over-expressing cells is mainly mediated by the enhanced formation of inositol 1,4,5-trisphosphate. This assumption is confirmed by the observation that the peak differences between control and hPLC-γ2 over-expressing cells were still apparent in the absence of extracellular Ca^{2+}. These data are similar to the recent findings of Renard et al. [14] which showed that over-expression of PLC-γ1 in NIH 3T3 cells induces an increased PDGF-mediated Ca^{2+} response. In contrast to these authors, however, we did not find a difference of the kinetic parameters of Ca^{2+} release in control and hPLC-γ2 over-expressing cells. This different observation may be due to a different coupling of PLC-γ1 and PLC-γ2 to the PDGF receptor and IP_3-stimulated Ca^{2+} release, or to the fact that the Ca^{2+} measurements presented in this paper result from about 10^3 attached cells whereas Renard et al. used single attached cells for their experiments.

There are several mechanisms by which hPLC-γ2 may exert its effect on the liberation of AA from membrane lipids. Activation of hPLC-γ2 by PDGF BB has been shown to induce the formation of inositol 1,4,5-trisphosphate [2] and an increase of intracellular Ca^{2+} (Fig. 5); simultaneously diacylglycerol is formed, which serves as an endogenous activator of PKC [15]. Ca^{2+} and PKC are both reported to mediate the activation of a phospholipase A_2 which liberates AA from membrane lipids [3–5]. Therefore, an hPLC-γ2-induced elevated level of intracellular Ca^{2+} may result in an enhanced stimulation of phospholipase A_2; additionally, the hPLC-γ2-induced elevated formation of diacylglycerol may lead to a more pronounced activation of PKC, which also results in an enhanced stimulation of phospholipase A_2. On the other hand, AA is reported to be liberated also by a phospholipase A_2-independent pathway, namely from diacylglycerol by a diacylglycerol lipase [16,17]. Therefore, the observed increased PDGF BB-mediated AA release in hPLC-γ2 over-expressing cells may also arise from AA liberated by a diacylglycerol lipase from diacylglycerol generated by hPLC-γ2. Further experiments including the quantification of diacylglycerol in control and hPLC-γ2-over-expressing cells are necessary to clarify which pathways are involved in AA release in hPLC-γ2 over-expressing NIH 3T3 cells.

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REFERENCES

[1] Ohta, S., Matsui, A., Nazawa, Y. and Kagawa, Y. (1988) FEBS Lett. 242, 31–35.
[2] Totzke, F., Marmé, D. and Hug, H. (1992) Eur. J. Biochem. 203, 633–639.
[3] Dennis, E.A., Rhee, S.G., Billah, M.M. and Hannun, Y.A. (1991) FASEB J. 5, 2068–2077.
[4] Felder, C.C., Dieter, P., Kinsella, J., Tamura, K., Kanterman, R.Y. and Axelrod, J. (1990) J. Pharmacol. Exp. Ther. 255, 1140–1147.
[5] Axelrod, J. (1990) Biochem. Soc. Trans. 18, 503–507.
[6] Fulton, A.M., Zhang, S. and Chong, Y.C. (1991) Cancer Res. 51, 2047–2050.
[7] Ongràdi, J. and Telekes, A. (1990) Acta Virol. 34, 380–400.
[8] Higgs, G.A. (1986) Prog. Lipid Res. 25, 555–556.
[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] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[12] Fain, J.N. (1990) Biochim. Biophys. Acta 1053, 81–88.
[13] Meldrum, E., Parker, P.J. and Carowi, A. (1991) Biochim. Biophys. Acta 1092, 49–71.
[14] Renard, C., Bolton, M.M., Rhee, S.G., Margolis, B.L., Zilberstein, A., Schlessinger, J. and Thomas, A.P. (1992) Biochem. J. 281, 775–784.
[15] Kikkawa, U. and Nishizuka, Y. (1986) Annu. Rev. Cell Biol. 2, 149–178.
[16] Burgoyne, R.D. and Morgan, A. (1990) Trends Biochem. Sci. 15, 365–366.
[17] Allen, A.C., Gammon, C.M., Ousley, A.H., McCarthy, K.D. and Morell, P. (1992) J. Neurochem. 58, 1130–1139.