FEBS Letters 347 (1994) 63–68

FEBS 14122

Selective nuclear translocation of protein kinase C α in Swiss 3T3 cells treated with IGF-I, PDGF and EGF

Luca M. Neri[a,d,*], Anna Maria Billi[b], Lucia Manzoli[c], Silvia Rubbini[d], R. Stewart Gilmour[e], Lucio Cocco[b], Alberto M. Martelli[f]

[a]*Istituto di Anatomia Umana Normale, Università di Ferrara, Ferrara, Italy*
[b]*Istituto di Anatomia Umana Normale, Università di Bologna, Bologna, Italy*
[c]*Istituto di Morfologia Umana Normale, Università di Chieti, Chieti, Italy*
[d]*Istituto di Citomorfologia Normale e Patologica del CNR, Chieti/Bologna (c/o IOR), Italy*
[e]*Dipartimento di Morfologia Umana Normale, Università di Trieste, Trieste, Italy*
[f]*Department of Cellular Physiology, AFRC Brabham Institute, Cambridge, UK*

Received 4 May 1994

Abstract

To determine the subcellular distribution of PKC after GFs treatment we have employed a combined immunochemical and in situ confocal microscopy analysis. In quiescent Swiss 3T3 cells only a faint PKC positivity was observable in the nucleus while a strong reaction was seen in the cytoplasm. IGF-I and to a lesser extent PDGF and EGF induced, after 45 min of treatment, a nuclear translocation of PKC detected by a pan-anti-PKC antibody and nuclear fluorescence was distributed in the nuclear interior except for the nucleolar regions. Bombesin and FGF did not affect the sub-cellular distribution of the enzyme. To further the understanding of which PKC isoform was involved in the translocation process, we have tested nine isozyme-specific anti-PKC antibodies. Immunoblotting analysis revealed the presence in Swiss 3T3 fibroblasts of α, β_1, ε and ζ isoforms. In isolated nuclei from GF-exposed cells only the α isozyme was detected: immunostaining was very intense after IGF-I treatment and clearly observable after PDGF and EGF stimulation. This result was strongly supported by the in situ confocal microscopy which parallels the Western blot analysis. These data demonstrate that several, but not all, GFs acting through tyrosine kinase receptor induce the intranuclear translocation of PKC α and, because of the dramatic effect of IGF-I, strengthen the case for a link between the activation of nuclear inositol lipid cycle and PKC translocation induced by this GF.

Key words: Protein Kinase C; Nucleus; Translocation; Confocal microscopy; Growth factor

1. Introduction

In mammalian cells, polypeptide factors trigger the generation of signals leading to cell growth and differentiation. Several GF receptors possess a protein tyrosine kinase activity, which acts as an on-off switch, inducing a phosphorylative cascade in the cytoplasm [1]. This phosphorylative cascade reaches the nucleus and while many nuclear proteins appear to be targets, the mechanism(s) responsible for the activation of DNA synthesis remain obscure [2,3]. Recently an entirely separate nuclear phosphoinositide signalling system has been demonstrated in Swiss 3T3 cells which is under the control of IGF-I receptor whose stimulation increases the activity of PLC β and the production of DAG [4–6]. In the same time frame, exposure of resting Swiss 3T3 cells to IGF-I induces translocation of PKC to the nucleus [5,6]. Therefore, it seems likely that nuclear DAG production is responsible for the translocation of PKC from the cytoplasm to the nucleus. However, it is still unclear whether this translocation is limited to the nuclear envelope or whether it is truly within the nucleus [5,7], not withstanding a number of reports which propose a nuclear localisation of PKC upon treatment with different agonists [8–10]. To solve such an issue we have employed confocal laser scanning microscopy to examine the intracellular distribution of PKC in Swiss 3T3 fibroblasts treated with different growth factors that are known to be physiological inducers of proliferation in 3T3 cells. We demonstrate that some but not all of these agonists, acting through receptors with an intrinsic tyrosine kinase activity, determine translocation of PKC within the nucleus. To identify which PKC isoform was involved in this phenomenon, we have employed nine isozyme-specific anti-PKC antibodies. The immunochemical analysis indicates that among the four PKC isozymes expressed in whole 3T3 cells, only the isoform α is selectively translocated to the nucleus after GFs treatment.

* Corresponding author. Istituto di Anatomia Umana Normale, Via Fossato di Mortara, 66, I-44100 Ferrara, Italy. Fax: (39) (51) 251735.

Abbreviations: PKC, protein kinase C; GF, growth factor; IGF-I, insulin-like growth factor I; PDGF, platelet-derived growth factor, EGF, epidermal growth factor; FGF, fibroblast growth factor; BSA, bovine serum albumin; PLC, phospholipase C specific for inositol lipid; DAG, diacylglycerol; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

SSDI 0014-5793(94)00508-S

2. Materials and methods

2.1. Source of materials, immunochemicals and purified PKC

All tissue culture reagents were from Sigma Chemical Co. (St. Louis, MO). All growth factors were from Boehringer (Mannheim, Germany). Anti-β tubulin antibody reacting with the β-Lc and β-sc fragments in the carboxy-terminus of β tubulin [11] was obtained from Sigma. Both the production and characterisation of the pan-anti-PKC serum raised against a synthetic peptide of the C-terminal sequence of PKC have been essentially described in details by Martelli et al. [12] and a data base search using the above synthetic peptide indicates that the sequence shows 100% sequence homology to PKC α, 93% to PKC δ, 89% to PKC γ and 60% to PKC β_I. The affinity purified antibodies against PKC isoforms α, β_I, β_{II}, γ, δ, ε, η, ζ and θ were purchased from Santa Cruz Biotechnology, (Santa Cruz CA, USA) and used at a concentration of 0.25 μg/ml. According to the manufacturer's specifications all of the antisera recognise a sequence mapping at or within the carboxy terminus of PKC (i.e. a region belonging to the catalytic domain of the enzyme). Alkaline phosphatase-conjugated and TRITC-conjugated anti-rabbit IgG were from Sigma. PKC was purified from rat brain as previously described by Martelli et al. [13].

2.2. Cell culture

Stock cultures of Swiss 3T3 cells (from Dr. K. Brown laboratory, AFRC, Cambridge, UK) responsive to mitogenic stimulation [14] were passaged at 3-day intervals in Dulbecco's modified Eagle's medium, containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum, in a humidified 10% CO_2 atmosphere. Prior to stimulation, cells were subcultured at a density of 10^4/cm^2 and incubated until they became confluent and quiescent (6–8 days).

Quiescent cultures of Swiss 3T3 cells were washed twice with serum-free medium containing 0.2% BSA and incubated in the same medium containing growth factors as follows: Bombesin (10 nM), IGF-I (20 ng/ml), PDGF (10 ng/ml), EGF (20 ng/ml), FGF (20 ng/ml).

2.3. Immunochemical analysis on both intact cells and isolated nuclei

Isolated nuclei were prepared exactly as previously reported [6]. It is worth recalling that these nuclei have been shown to be free from cytoplasmatic contamination by means of both biochemical and morphological criteria [6]. Moreover, immunochemical analysis with anti-β tubulin monoclonal antibody used at 1:500 dilution, reacted with alkaline phosphatase-conjugated anti-mouse IgG as secondary antibody and detected as detailed below, demonstrated the absence of this cytoskeletal component (see section 3). Swiss 3T3 cells were directly dissolved in hot electrophoresis sample buffer and the lysates subsequently were repeatedly passaged through a 22-gauge needle to shear chromosomal DNA and reduce viscosity. Cell and nuclear proteins were separated on 7.5% polyacrylamide/0.1% sodium dodecylsulfate gel and blotted to 0.22 μm nitrocellulose membrane. Strips containing individual lanes were saturated with PBS containing 5% BSA for 1h at 37°C and then incubated for 2 h at room temperature with a 1:100 dilution of the pan anti-PKC serum [12] in PBS, 10% normal goat serum, 3% BSA or with affinity purified antibodies against the following PKC isoforms: α, β_I, β_{II}, γ, δ, ε, η, ζ and θ. The membrane strips were washed five times with PBS, 0.1% BSA and then incubated for 1 h at room temperature with a 1:500 dilution of an alkaline phosphatase-conjugated anti-rabbit IgG in PBS, 0.1% BSA. After washing as above, antibody binding was detected using Nitrotetrazolium blue and bromo- chloro-indolyl phosphate as substrates.

2.4. Detection of PKC by in situ immunofluorescence and confocal microscopy

Cells were washed twice in cold PBS, pH 7.2 and processed for indirect immunofluorescence using fixation with freshly made 4% paraformaldehyde in PBS (30 min at room temperature) and permeabilised with 0.2% Triton X-100 in PBS (10 min). The pan-anti PKC polyclonal antibody raised against the catalytic region of PKC [12] was used at a dilution of 1:25 in 2% BSA, 3% normal goat serum in PBS. The secondary antibody was a TRITC-conjugated anti-rabbit IgG diluted 1:80. All incubations were carried out at 37°C. Finally the coverslips were mounted in glycerol containing 1,4-diazabicyclo[2.2.2]octane to retard fading, using additional coverslips as spacers to preserve the three-dimensional structure of cells. A variety of controls was performed to ensure the specificity of the antibody used: coverslips were treated with preimmune serum as well as with the secondary antibody alone. No fluorescence at all was seen (not shown). For conventional epifluorescence, specimens were viewed in a Zeiss Axiophot photo microscope and photographs were taken on Kodak T-Max film. Digital images were taken with a Phoibos 1000–Sarastro (Molecular Dynamics) confocal microscope coupled with a Nikon Optiphot and equipped with an external air cooled argon laser (Spectra Physics) emitting in a range of 457–514. The laser was tuned at a power output of 10 mV and the laser beam attenuated to 10% with neutral density filters. To obtain the highest possible resolution, a 63× planapo oil immersion objective was used (N.A. 1.4). TRITC was excited with a 514 nm bandpass and a 530 nm dichroic filter. Emission filter was a 590/15 nm bandpass. The theoretical optical section thickness was 0.6 μm. All images of a given cell were taken with the same photomultiplier value in a range of 750–950 V and sequential optical sections were 0.6 μm apart in the z axis. On the x–y axis the scanning mode format was 512 × 512, with a pixel size of 0.2. Digital images processing, quantitation and display were generated as previously described [15].

3. Results

3.1. Immunochemical analysis

Nuclear proteins from control (unstimulated) and growth factor-treated (45 min of stimulation) cells were separated by denaturing polyacrylamide gel electrophoresis, blotted onto nitrocellulose paper and probed for the presence of PKC by means of a polyclonal antibody raised against a synthetic peptide [12] with 100% sequence homology to PKC α, 93% to PKC δ, 89% to PKC γ and 60% to PKC β_I. Fig. 1A shows that nuclei from control cells and cells treated with Bombesin and FGF contained only a very limited amount of protein with a M_r of 80 kDa, comigrating with semipurified PKC from rat brain. On the other hand, nuclei isolated from cells exposed to IGF-I, PDGF and EGF displayed a higher immunoreactivity for PKC. However, the strongest immunochemical reaction for PKC was by far that seen in nuclei from IGF-I-stimulated cells. The PKC immunoreactivity did not change in lysates obtained from quiescent and agonist-treated cells (Fig. 1B), in which the antibody also detected a band migrating at 50 kDa.

The 50 kDa protein corresponds to the catalytic domain of PKC. Indeed, it should be recalled that PKC is cleaved by neutral proteases such as calpains in the V_3 hinge region to generate two fragments, the regulatory and the catalytic protein kinase domains, of which the latter contains the carboxy terminus of the enzyme [2,16]. We have previously shown that the pan-PKC antibody recognisis the catalytic domain of the kinase obtained by limited tryptic digestion [13]. Even if isolated nuclei fulfilled the biochemical and morphological criteria of purity [6], the blotted nuclear proteins from control and IGF-I treated cells, which induce the stronger nuclear translocation of PKC, were also probed for the presence of possible cytoplasmatic contamination by means of anti-β tubulin antibody. Indeed Fig. 1C shows that whilst whole cells display a strong immunoreactivity at 55 kDa with the anti-β tubulin antibody, in isolated nuclei β tubulin is completely absent.

The use of isozyme-specific antibodies revealed the expression in Swiss 3T3 cells of only four PKC isoforms:

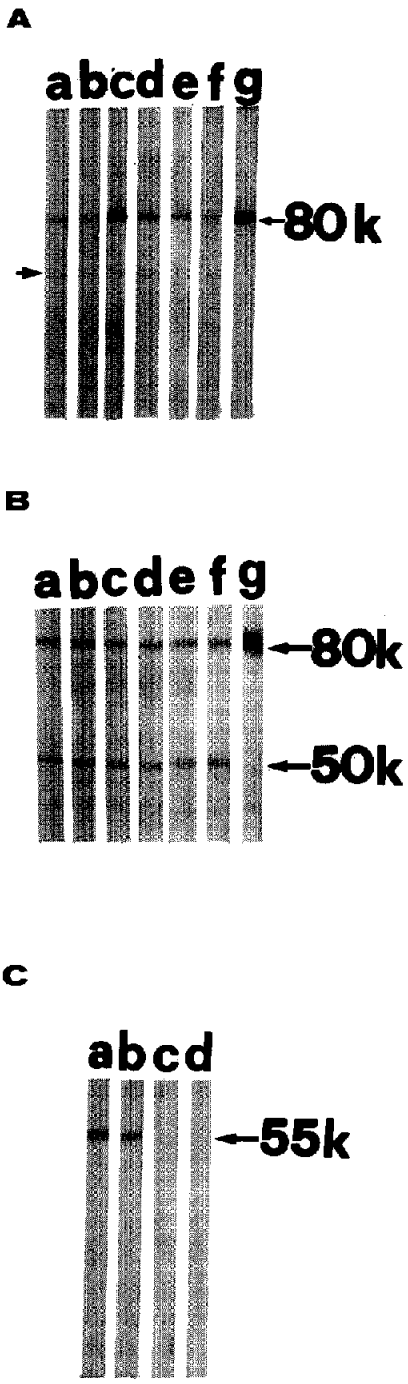

Fig. 1. Immunoblotting analysis by means of pan-anti-PKC antibody of Swiss 3T3 cells treated with GFs. Panel (A) isolated nuclei, panel (B) whole cells. Lane a, control cells; lane b, Bombesin-treated cells; lane c, IGF-I-treated cells; lane d, PDGF-treated cells; lane e, EGF-treated cells; lane f, FGF-treated cells; lane g, purified PKC from rat brain. Panel C shows the immunoblotting analysis with anti-β tubulin antibody on both purified nuclei and whole cells. Lane a, control whole cells; lane b, IGF-I treated whole cells; lane c, nuclei from control cells; lane d, nuclei from IGF-I treated cells. For each lane in (A), (B) and (C) 80 μg of protein was loaded. In panel (A) arrowhead points to a very faint 50 kDa band.

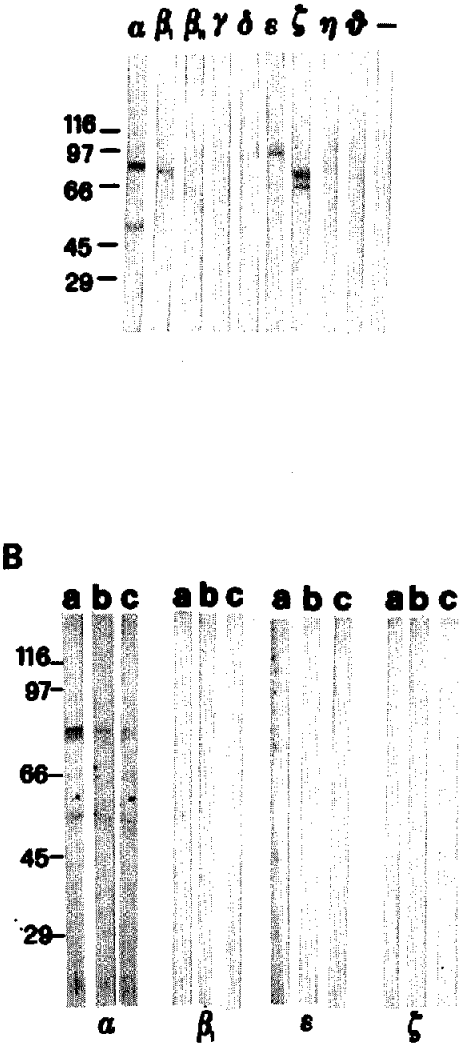

Fig. 2. Immunoblotting analysis by means of anti-PKC isozyme antibodies of Swiss 3T3 cells and subcellular fractions. Panel (A) whole resting cells, panel (B) isolated nuclei. In panel (A) Greek letters refer to the various isoforms and the hyphen on the far right lane indicates the reaction in the absence of any primary antibody. Panel (B): lanes a, b and c refer to nuclei from cells treated with IGF-I, PDGF and EGF, respectively probed with isozyme-specific anti-PKC-α, -β_I, -ε and -ζ antibodies. For each lane in both (A) and (B) 80 μg of protein was loaded.

α, β_I, ε, ζ (Fig. 2A). The position of the immunoreactive bands was consistent with the calculated molecular weight of these isoforms [2]. The immunochemical analysis by means of the above antibodies on nuclei isolated from cells treated with the GFs responsible for PKC translocation to the nucleus shows that after treatment with IGF-I, PDGF or EGF only the α isoform translocated to the nucleus (Fig. 2B). It should be noted that the antibody specific for the α isoform revealed that nuclear fractions contained both the native 80 kDa polypeptide

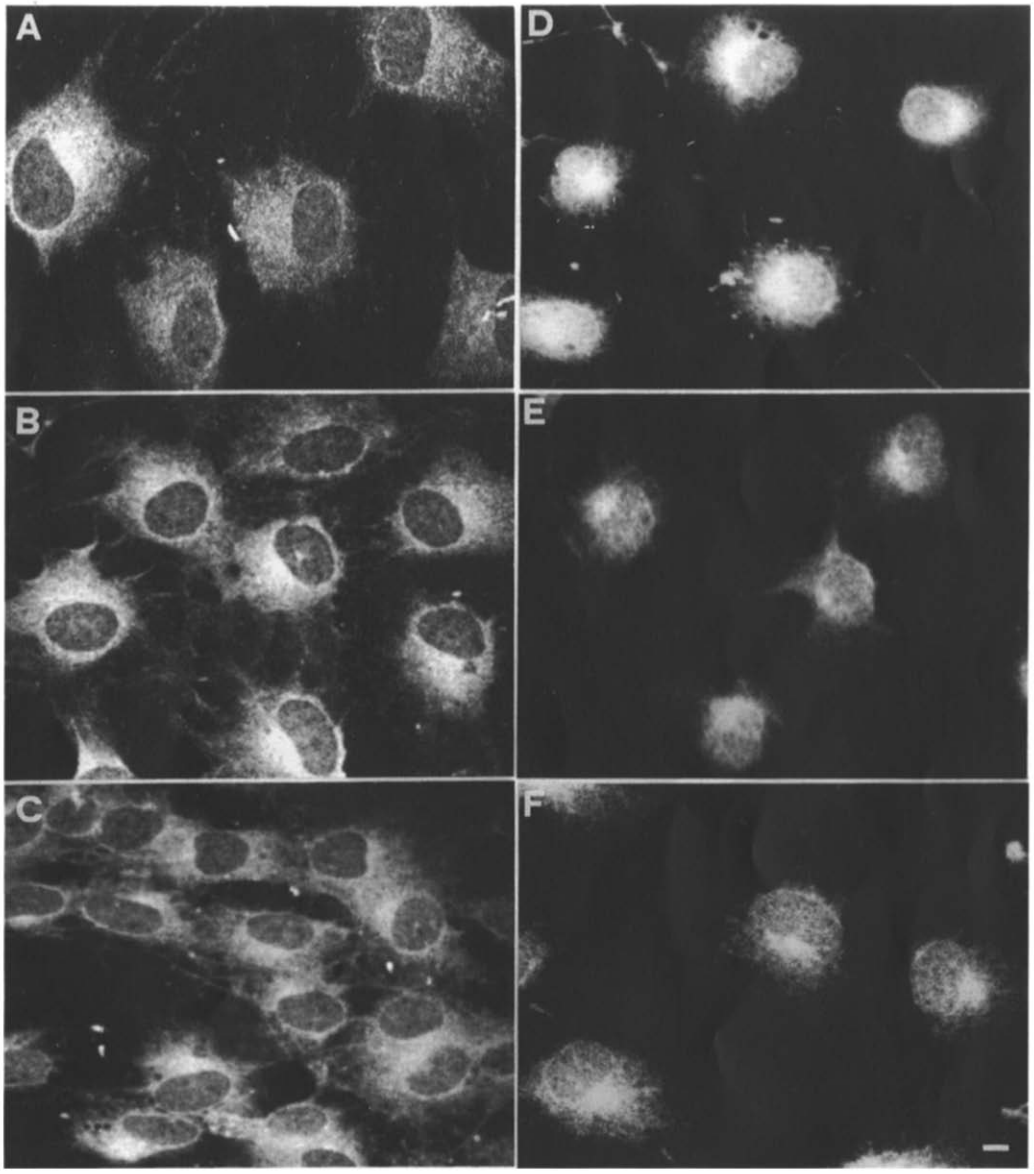

Fig. 3. Confocal laser scanning microscopy optical sections through the equatorial plane of Swiss 3T3 cells stained with the pan-anti-PKC antibody. A, control; B, Bombesin, C, FGF; D, IGF-I; E, PDGF; F, EGF. Bar = 5 μm.

and the 50 kDa proteolytically-cleaved fragment of PKC. From the same figure it clearly appears that IGF-I induces a massive translocation of the α isozyme whereas after PDGF and EGF treatment this occurs to a lower extent.

At about 80 kDa it is possible to observe a double band; this probably reflects different phosphorylation states of PKC α because this isoform is known to be phosphorylated mainly on serine residues which induces changes in its electrophoretic mobility [2,17]. Such a doublet of bands is not detected by the pan-PKC antiserum. However, it should be emphasized that the peptide sequences recognised by the two antibodies are different (i.e. the sequence of the synthetic peptide for the pan-PKC antibody is CYVNPQFVHPILQSAV whereas that for the PKC-α antiserum from Santa Cruz Biotechnology is DFEGFSYVNPQFVHPILQSSV). It might be that the latter antiserum can also recognise phosphorylated PKC. These phosphorylative events are currently under investigation in our laboratory in order to analyse the phosphoaminoacid pattern and their possible implications in the regulation of PKC activity.

3.2. *Confocal immunofluorescence and nuclear localization*

For the *in situ* analysis the pan PKC polyclonal antibody raised against the synthetic peptide with 100% sequence homology to PKC α was used. The choice was suggested because of the high reliability of the antibody for immunocytochemistry [12] and because the screening

with the isoform-specific antibodies indicated that only PKC α translocates to the nucleus. Swiss 3T3 fibroblasts were fixed, permeabilised and reacted with the pan-PKC polyclonal antibody and finally with TRITC-conjugated secondary antibody.

To rule out the possibility that treatment of the cells with GFs modifies PKC distribution in the cytoplasm above and below the nucleus, but not within the nucleus, an extensive laser scan confocal analysis has been performed. Optical sections through control cells showed a diffuse staining of the cytoplasm very similar to that observed with anti-cytoskeleton antibodies [18] with fluorescent fibres aligned parallel to the long axis of the cells and the filamentous network extending throughout the cell. In contrast to the strong labelling of the cytoplasm, a faint punctate fluorecence of the nuclear region was observable (Fig. 3A). Sometimes the edges of the nuclear membrane were also stained. The results obtained suggest that in control cells the antibody identified several distinct sites of PKC distribution and these included the cytoplasm along with its cytoskeletal component and the perinuclear region. Bombesin and FGF treatment did not alter the overall distribution of PKC which was detectable at very low levels within the nucleus (Fig. 3B,C). Incubation of cells with IGF-I, PDGF or EGF induced changes in the subcellular localisation of PKC as shown in Fig. 3 D, E and F, respectively. Exposure to these GFs for 45 min. caused a decrease in the cytoplasmic PKC levels, while labelling was increased in the nuclear region. However, the redistribution of PKC in Swiss 3T3 fibroblasts treated with these three GFs revealed some differences, being strongest in the case of IGF-I-treated cells compared with cells incubated in the presence of PDGF or EGF (Fig. 3D,E,F). The optical sections allowed us to analyse the nuclear interior without out-of-focus blurring of fluorescence, which unfortunately occurs in conventional fluorescence microscopy. Under these conditions nucleoli, appearing as black dots in the pictures, are unstained in all samples.

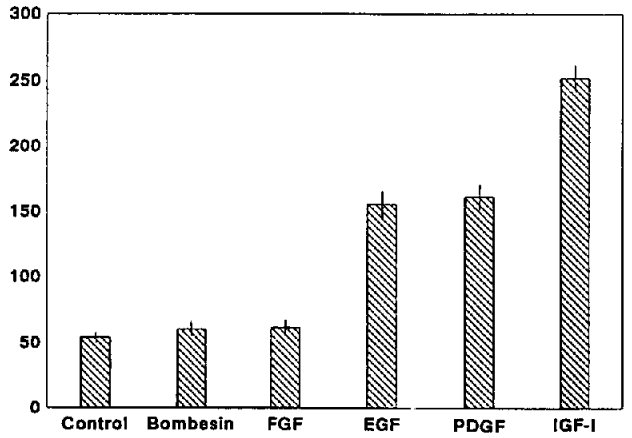

Fig. 4. Nuclear fluorescence intensity evaluated by the 'extended focus' technique [12]. The values are the average of five separate determinations ± S.D. and are expressed as arbitrary units obtained by an image processing that analyses the fluorescence of the three-dimensional reconstruction of the whole cell in confocal microscopy.

The nuclear fluorescence intensity, evaluated by the 'extended focus' technique [15], is depicted in Fig. 4, which shows that while IGF-I treatment caused a five-fold increase in the staining PDGF and EGF caused only a three-fold increase. Bombesin and FGF did not significantly affect the nuclear fluorescence signal.

4. Discussion

The PKC family of isoenzymes is part of a complex network of signal transduction pathways involving both GTP-binding proteins and growth factor receptor tyrosine kinases (for review see [19]).

Here we show that GFs acting through receptors with intrinsic tyrosine kinase activity, namely IGF-I, PDGF and EGF, induce nuclear translocation of PKC α, whereas Bombesin and FGF, which stimulate polyphosphoinositide hydrolysis at the plasma membrane [19], do not. The nuclear translocation of PKC has been shown to occur after treatment with phorbol ester or bryostatin [8,10], however the presence of PKC has been reported in nuclei of different cells also in the absence of a mitogenic stimulus [20,21]. Among PKC isoforms, the α isoform has been shown to translocate to the nucleus after phorbol ester treatment of NIH 3T3 cells [9] or HL-60 cells [22], while the β isoform seems to reside in the nucleus of resting hepatocytes [23]. The coupling of DAG generation [5,6] and translocation of a specific PKC isoform (i.e. PKC α) provides a mechanism for explaining previously published observations [4,12]. Moreover, confocal microscopy resolves the controversial issue of the actual localisation of PKC in the nucleus after translocation. Indeed, in combination with the immunocytochemical analysis the *in situ* confocal sections through the equatorial plane of the cells (Fig. 3) indicated that some GFs (i.e. IGF-I, PDGF and EGF) induced an accumulation of PKC α within the nucleus. IGF-I, which activates nuclear PLC β and increases the concentration of nuclear DAG [5,6], gave rise to the highest level of translocation. This suggests that DAG acts as an attractant for PKC α and this finding is strengthened by the absence of PKC α translocation to the nucleus in the cells stimulated with Bombesin, which does not induce at all any increase in nuclear DAG levels [5]. It will be of interest to study whether or not PDGF and EGF can affect nuclear polyphosphoinositide hydrolysis. Our data contribute to an understanding of how signals originating at the plasma membrane are transmitted to the nucleus. The steps leading to mitosis are characterised by an ordered sequence of events which link the nuclear inositol lipid cycle to the translocation and activation of PKC α through the accumulation of nuclear

DAG, a well-known physiological activator of this isoform [24]. In addition to the phosphorylation of nuclear lamins [25], PKC could also phosphorylate other nuclear proteins some of which may be involved in the early nuclear events leading to DNA replication. Finally, it is interesting that the use of isozyme-specific antisera revealed that in Swiss 3T3 cells only PKC α occurs in both the native and proteolitically-cleaved forms. Indeed, it is known that other isoforms (i.e. PKC β, γ and ε) can be cleaved by calpains [2]. Further work will be necessary to determine the role of proteolytic cleavage with respect to PKC α activation and relocalisation to cellular compartments.

Acknowledgments: Authors are grateful to A. Fantazzini and to A. Valmori for skilful photographic assistance. This work was supported by Italian CNR Grants 93.01872.CT12, PF ACRO and PF IG.

References

[1] Iwashita, S. and Kobayashi, M. (1992) Cell. Signall. 4, 123–132.
[2] Hug, H. and Sarre, T.F. (1993) Biochem. J. 291, 329–343.
[3] Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Cell 64, 281–302.
[4] Manzoli, F.A., Martelli, A.M., Capitani, S., Maraldi, N.M., Rizzoli, R., Barnabei, O. and Cocco, L. (1989) Adv. Enzyme Regul. 28, 24–34.
[5] Divecha, N., Banfic', H. and Irvine, R.F. (1991) EMBO J. 10, 3207–3214.
[6] Martelli, A.M., Gilmour, R.S., Bertagnolo, V., Neri, L.M., Manzoli, L. and Cocco, L. (1992) Nature 358, 242–245.
[7] Banfic', H., Žizak, M., Divecha, N. and Irvine, R.F. (1993) Biochem. J. 290. 633–636.
[8] Thomas, T.P., Talwar, H.S. and Anderson, W.B. (1988) Cancer Res. 48, 1910–1919.
[9] Leach, K.L., Powers, E.A., Ruff, V.A., Jaken, S. and Kaufmann, S. (1989) J. Cell Biol. 109, 685–695.
[10] Hocevar, B.A., Morrow, D.M., Tykocinski, M.L. and Fields, A.P. (1992) J. Cell Sci. 101, 671–679.
[11] Matthes, T., Wolff, A., Soubiran, P., Gros, F. and Dighiero G. (1988) J. Immunol. 141, 3135–3141.
[12] Martelli, A.M., Neri, L.M., Gilmour, R.S., Barker, P.J., Huskisson, N.S., Manzoli, F.A. and Cocco, L. (1991) Biochem. Biophys. Res. Commun. 177, 480–487.
[13] Martelli, A.M., Carini, C., Marmiroli, S., Mazzoni, M., Barker, P.J., Gilmour, R.S. and Capitani, S. (1991) Exp. Cell Res. 195, 255–262.
[14] Martelli, A.M., Gilmour, R.S., Neri, L.M., Manzoli, L., Corps, A.N. and Cocco, L. (1991) FEBS Lett. 283, 243–246.
[15] Martelli, A.M., Neri, L.M., Zamai, L., Bareggi, R., Manzoli, L. and Cocco, L. (1994) Histochem. J. 26, 179–188.
[16] Eldar, H., Ben-Chaim, J. and Livneh, E. (1992) Exp. Cell Res. 202, 259–266.
[17] Pears, C., Stabel, S., Cazaubon, S. and Parker, P.J. (1992) Biochem. J. 283, 515–518.
[18] Murti, K.G., Kaur, K. and Goorha, R.M. (1992) Exp. Cell Res. 202, 36–44.
[19] Berridge, M.J. (1993) Nature 361, 315–325.
[20] Wood, J.G., Girard, P.R., Mazzei, G.J. and Kuo, J.F. (1986) J. Neurosci. 6, 2571–2577.
[21] Capitani, S., Girard, P.R., Mazzei, G.J., Kuo, J.F., Berezney, R. and Manzoli, F.A. (1987) Biochem. Biophys, Res. Commun. 142, 367–375.
[22] Hocevar, B.A. and Fields, A.P. (1991) J. Biol. Chem. 266, 28–33.
[23] Rogue, P., Labourdette, G., Masmoudi, A., Yoshida, Y., Huang, F.L., Huang, K.-P., Zwiller, J., Vincendon, G. and Malviya, A.N. (1990) J. Biol. Chem. 265, 4161–4165.
[24] Nishizuka, Y. (1988) Nature 334, 661–665.
[25] Irvine, R.F. and Divecha, N. (1992) Semin. Cell Biol. 3, 225–235.