Biochemical Pharmacology, Vol. 53, pp. 1413–1418, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00863-5

Cellular Relocalisation of Protein Kinase C-θ Caused by Staurosporine and Some of Its Analogues

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ABSTRACT. The microbial product staurosporine is a protein kinase C (PKC) inhibitor with some phorbol ester-agonistic properties. It is known to cause the translocation of the PKC isoenzymes ε and δ from the cellular cytosol to the membrane and nucleus. We tested the hypothesis that it also affects the cellular localisation of the novel PKC isoenzyme θ, and that staurosporine analogues, some of which are currently under clinical evaluation as potential anticancer drugs, have a similar effect. Their ability to alter PKC-θ distribution was studied in human-derived A549 lung carcinoma cells. Western blot analysis and confocal microscopy after indirect immunofluorescence staining showed that staurosporine (100 nM), like the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (25 nM) caused the translocation of PKC-θ from the cytosol to the membrane and the nucleus. The bisindolylmaleimide GF 109203X mimicked staurosporine, but had a weaker effect. Ro 31-8220 and UCN-01 decreased cytosolic PKC-θ only at 1 μM. CGP 41251 had no effect on PKC-θ in either experimental design. The results show that some, but not all, staurosporine analogues share the partial phorbol ester-agonistic PKC-translocatory activity of the parent molecule. BIOCHEM PHARMACOL 53;10:1413–1418, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. bisindolylmaleimides; protein kinase C translocation; staurosporine

The enzyme family protein kinase C (PKC†) plays a pivotal role in signal transduction pathways that influence cell proliferation and differentiation [1, 2]. PKCs are the major cellular receptors for tumour-promoting phorbol esters, and are therefore thought to play a major role in carcinogenesis. Phorbol esters bind directly to and stimulate the catalytic activity of PKCs. In many cell types PKC activation is intrinsically associated with its redistribution from the cytosol to the membrane and/or nucleus, the *loci* of physiologically important targets of PKC-catalysed phosphorylations. PKC comprises a multigene family that encodes at least 12 distinct isoforms [3]. According to cofactor requirements for activity and responsiveness towards activation by phorbol esters, PKC can be divided into three subfamilies. The "conventional" PKCs (cPKCs), such as PKC-α, need calcium for maximal activity, the "novel" PKCs (nPKCs), for example PKC-δ, -θ and -ε, are calcium independent, and the "atypical" PKCs (aPKCs), such as PKC-ζ, are not activated by phorbol esters or diacylglycerols. The differences between the distinct PKC isoenzymes with respect to cofactor dependency, subcellular localisation, tissue distribution, and levels of expression in a given cell type suggest that they may be independently regulated via coupling to distinct receptor signalling pathways, and possess different, albeit overlapping, substrate specificities. The microbial product staurosporine is a potent inhibitor of protein kinases, a weak tumour promotor in mouse skin and a fairly potent inhibitor of phorbol ester-induced tumour promotion [4, 5]. Staurosporine blocks phorbol ester responses in neutrophils, but not in keratinocytes, where it displays some PKC-agonistic characteristics [6, 7]. It also behaves like a PKC activator in that it causes the cellular redistribution of nPKCs-δ and -ε [8]. Staurosporine has served as a "lead compound," and a series of analogues have been synthesised, some of which display greater selectivity for PKC over other kinases, even though they are somewhat less potent as inhibitors than staurosporine [9]. Examples of such analogues are the indolocarbazoles 4′-hydroxystaurosporine (UCN-01) and *N*-benzoylstaurosporine (CGP 41251) and the bisindolylmaleimides Ro 31-8220 and GF 109203X (see Fig. 1 for structures). UCN-01 and CGP 41251 possess antineoplastic activity in rodent tumour models [10, 11], and they are currently in phase I clinical trial as potential anticancer drugs. Some of the staurosporine analogues, like the parent compound, cause the cellular redistribution of PKC-ε [12]. In this work we focus on PKC-θ, a nPKC isoenzyme that was characterised relatively recently and that displays a unique tissue distribution, with prevalence in skeletal muscle, lymphoid organs, and haematopoeitic cell lines [13–15]. However, very little information exists with regard to its functional and biochemical properties. The purpose of the study described here was to determine if staurosporine and its analogues affect the cellular localisation of PKC-θ in a similar manner

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† *Abbreviations:* PKC: protein kinase C; RACKs: receptors for activated C kinase.

Received 28 August 1996; accepted 14 November 1996.

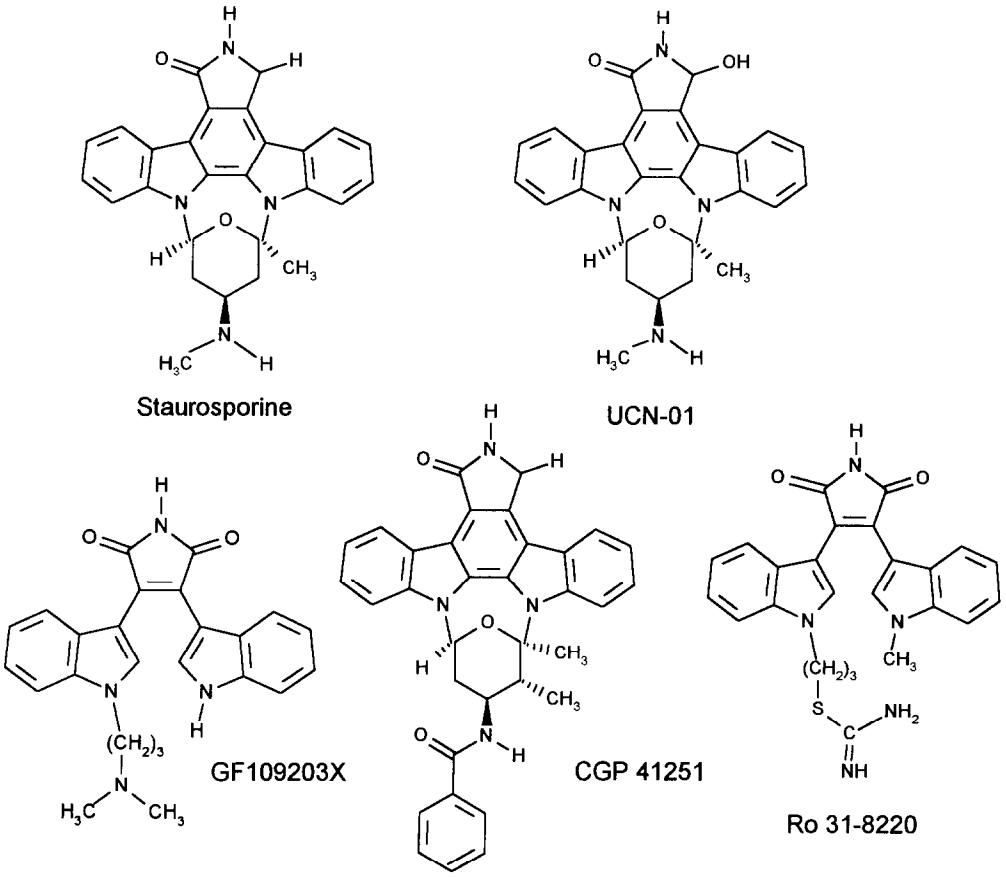

FIG. 1. Structures of staurosporine and the analogues studied in this article.

to that of PKC-ε. In this way we wished to test the hypothesis that the ability of kinase inhibitors to cause PKC translocation extends to nPKCs in general, rather than to one isozyme only.

MATERIALS AND METHODS

Drugs and Reagents

UCN-01, CGP 41251, and Ro 31-8220 were provided by Kyowa Hakko Kogyo (Tokyo, Japan), Ciba Geigy (Basle, Switzerland) and Roche Research Centre (Welwyn Garden City, UK), respectively. GF 109203X was acquired from Calbiochem-Novabiochem (Nottingham, UK). Staurosporine and other drugs and reagents were purchased from Sigma (Poole, UK). Stock solutions of compounds were prepared in dimethyl sulfoxide (DMSO). The monoclonal antibody against PKC-θ, raised against a 25.3 kDa protein fragment corresponding to residues 21–217 in the regulatory domain of mouse PKCq as immunogen, was purchased from Affiniti Research Products Ltd. (Nottingham, UK).

Cell Growth

Human-derived A549 lung adenocarcinoma cells were obtained from the European Collection of Animal Cell Culture (Salisbury, UK). Cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum (Globe Pharm Ltd., Esher, UK), penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (2 mM) in an atmosphere of 5% carbon dioxide. Cells were subcultured routinely twice a week.

Preparation of Cellular Fractions and Western Blot Analysis

Subconfluent cells were incubated with TPA (25 nM), staurosporine, UCN-01, CGP 41251, GF 109203X, or Ro 31-8220 (10 nM to 1 μM for staurosporine, 50 nM to 1 μM for the others). Compounds were dissolved in DMSO, the final DMSO concentration was 0.3% at which cell growth was not affected. After incubation for 30 min cytosolic, particulate (which contains membranes, cytoskeleton, and cell organelles) and nuclear fractions were obtained essentially as described before [16] with some modifications [17]. Protein content of the fractions was measured by the Bradford assay [18]. Western blot analysis for PKC-θ was performed as described previously [17]. The amount of protein loaded was 20 μg per lane. Detection was by enhanced chemiluminescence generated by oxidation of luminol in the presence of hydrogen peroxide using a kit from Amersham International (Amersham, UK). Immuno-

reactivity was quantitated using a Molecular Dynamics Computing Densitometer (using Image Quant 3.0 software), and the values shown in Fig. 2B are percentages of the sum of band volume of enzyme in all three fractions. The molecular weight of the detected protein was 79 kDa. An immunogen to compete away PKC-θ immunoreactivity was not available. We ascertained the specificity of the antibody by establishing that it did not cross-react with bands that, in control experiments, immunoreacted with antibodies against cPKCs or nPKCs-ε and -δ.

Immunofluorescence Staining and Confocal Microscopy

Cells were grown on coverslips until confluent and incubated with TPA, staurosporine, or its analogues (dissolved in DMSO to yield a final concentration of 0.05% in the incubate) for 30 min. Cells were then fixed with formaldehyde (3% in PBS, 10 min), and permeabilised with 0.05% Triton X-100 in PBS for 10 min. Cells were incubated with PKC-θ antibody (0.1 mg protein/ml) for 12 hr at room temperature. They were labelled with fluorescein-linked antimouse immunoglobulin G (0.1 mg protein/ml in PBS) for 1 hr at 37°C. Nonspecific binding was controlled by inclusion in the antibody solution of 20% sheep serum as blocking agent. Nuclei were counterstained with propidium iodide (PI, 1.25% in PBS) for 5 min at 4°C, and the coverslips were mounted using Fluoromount (BDH, Lutterworth, UK).

A Leica True Confocal Scanner 4D was used to identify subcellular localisation of enzyme. The microscope was configured to scan simultaneously in two channels, at 488 and 568 nm, to excite fluorescein and propidium iodide, respectively. Throughout the experiments levels of laser emission were kept the same for all samples and both photomultiplier tube settings were kept constant. The respective emissions were detected and cell preparations were optically sectioned. Optical data stacks were combined into a maximum projection and stored as true colour 24-bit digital images. Each dual-channel image was checked for "crosstalk" using Leica multicolour analysis software, and the images were corrected, when necessary. This procedure ensured that none of the green signal (FITC) was contaminated by the red signal (PI), or *vice versa*. The digital images were imported into Adobe Photoshop software and colour output was obtained using a Kodak dye sublimation printer.

RESULTS

Cells were incubated with staurosporine and its analogues for 30 min, and PKC-θ was detected by Western blotting in the cytosol, membrane, and nucleus. In untreated cells PKC-θ was mainly localised in the cytosol (46% of total enzyme) and the membrane (41%), and little (14%) was found in the nucleus. In control experiments TPA caused enzyme translocation as expected, predominantly to the membrane (Fig. 2A). Of the kinase inhibitors examined

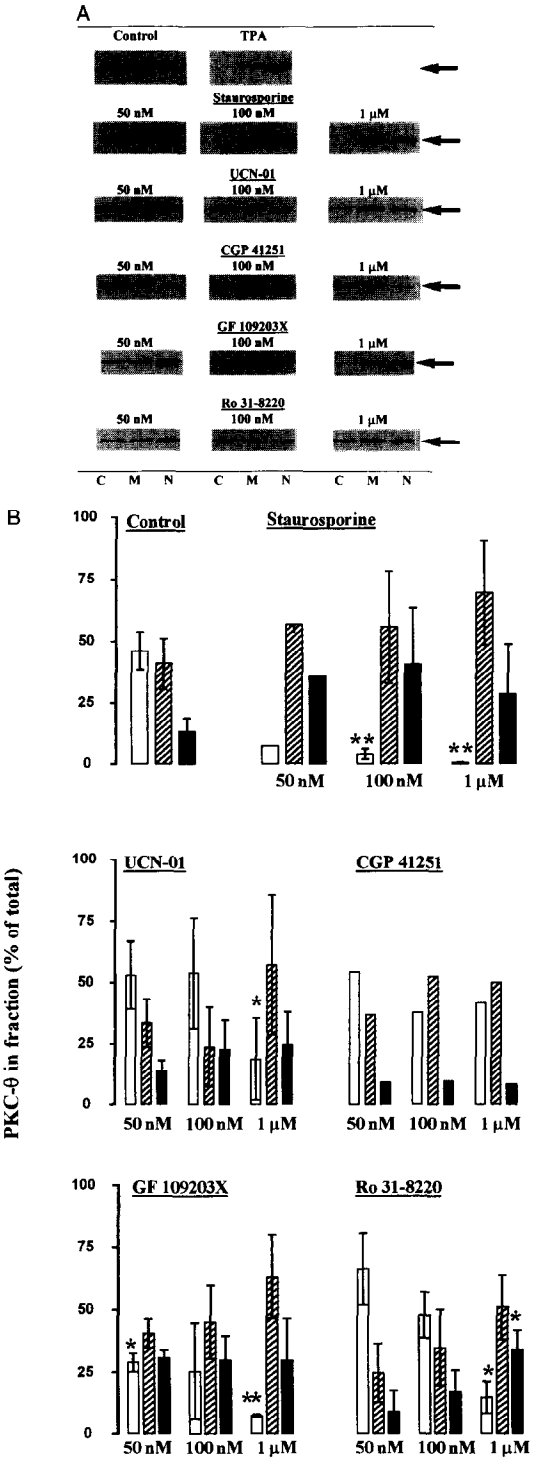

FIG. 2. Western blot analysis (A), and their quantitative evaluation by laser densitometry (B), of PKC-θ in the cytosol (C and open bars), membranes (M and hatched bars), and nuclei (N and closed bars) of A549 cells exposed to TPA (only in A), staurosporine, UCN-01, CGP 41251, Ro 31-8220, and GF 109203X. Cells were incubated with agents for 30 min. Blots in A are representative of two or three experiments, values in B are the mean of two or the mean ± SD of three experiments. Stars indicate that the difference between the value and the equivalent one in control cells is significantly different (**$P < 0.05$; Students' *t*-test), or approaching significance (*$P < 0.1$). For details of cell fractionation and Western analysis, see Materials and Methods.

staurosporine was the most potent redistributor of PKC-θ from the cytosol to both the membrane and nucleus (Fig. 2). At 100 nM staurosporine decreased cytosolic levels to less than 10%, with 56 and 34%, respectively, localised in the membrane and nucleus. GF 109203X translocated PKC-θ more potently than the other three staurosporine analogues, but not as effectively as staurosporine. At 50 nM it decreased cytosolic levels to 29% and increased nuclear levels to 30%, without causing a change in membrane PKC. Ro 31-8220 and UCN-01 both had only a weak effect. At 100 nM they did not change enzyme distribution, but at 1 μM Ro 31-8220 decreased cytosolic enzyme to 15%, and UCN-01 diminished it to 19%. At the same time Ro 31-8220 increased nuclear PKC-θ to 35%. In the case of UCN-01 we cannot exclude the possibility that it caused a reduction of PKC-θ in the cytosol without an increase in the other two fractions. CGP 41251 did not alter cellular levels of PKC-θ at all.

PKC-θ distribution was also investigated in intact cells using confocal microscopy. Indirect immunostaining showed that in the control cells expression of PKC-θ occurred mainly in the cytosol and membrane, as adjudged by green fluorescence (Fig. 3A), which is consistent with the results obtained by Western blot. TPA (25 nM) caused enzyme translocation mainly to the membrane, but also to the nuclear region (Fig. 3B). Staurosporine (1 μM) mimicked the effect of TPA (Fig. 3C). In the treated cells the nuclei appear yellow (Fig. 3B and C) compared to their red coloration in the control cells (Fig. 3A), which indicates an increased percentage of green-labelled PKC-θ in the nuclei superimposing the nuclear red staining by PI. GF 109203X (Fig. 3D) and Ro 31-8220 (Fig. 3E) (both 1 μM) had a weak translocatory effect, rendering staining in the cytosol more punctate. PKC-θ distribution in cells treated with UCN-01 (Fig. 3F) and CGP 41251 (not shown) (both 1 μM) was not consistently different from that in control cells when measured by this method.

The enzyme translocation observed with staurosporine and GF 109203X did not lead to enzyme downregulation as adjudged by Western analysis after incubation for 24 hr, in the manner achieved by long-term treatment with TPA (result not shown). However, exposure of the cells to UCN-01 or RO 31-8220 for 6 months caused a drastic decrease in cellular PKC-θ levels (C. Courage, S. Bradder, T. Jones, M.-H. Schultze-Mosgau and A. Gescher, manuscript submitted).

DISCUSSION

Recent advances in the pharmacology of the PKC system have furnished a variety of small molecules that modulate PKC activity, levels, and distribution with some degree of isoenzyme selectivity. Among them are the microbial product staurosporine and its analogues. Staurosporine, a non-specific kinase inhibitor, causes the redistribution of nPKCs-δ, -ε [8], and as shown above, nPKC-θ, but not of cPKCs or aPKCs. The staurosporine analogues UCN-01, CGP 41251, Ro 31-8220, and GF 109203X inhibit PKC much more selectively than staurosporine [9, 19, 20] and cPKCs more potently than nPKCs [21, 22]. Of these analogues UCN-01, Ro 31-8220 and GF 109203X at high concentrations, like staurosporine, redistribute nPKC-ε [12]. We describe here for the first time that staurosporine and GF 109203X change the cellular distribution of nPKC-θ, and that UCN-01 and Ro 31-8220 have a weak effect on cytosolic levels of this isoenzyme. Even though these phenomena may be cell-type-specific, this and other similar studies indicate that an array of agents is now available to the pharmacologist, which allows some fine-tuning of the activity and cellular localisation of PKC in an isoenzyme-specific fashion.

The results presented above together with the relevant literature suggest that the redistributory ability of staurosporine, UCN-01, Ro 31-8220, and GF 109203X extends to all nPKC isoenzymes, as it has been shown previously to apply to PKC-δ and -ε [8, 12], and now to PKC-θ. Furthermore, our results indicate that there is little difference between staurosporine analogues in terms of the direction into which they translocate PKC-θ, that is to both membrane and nuclei. The difference in nPKC-translocatory activity between staurosporine, CGP 41251, and UCN-01 is intriguing. Introduction of a benzoyl function on the nitrogen in position 4′ of the staurosporine glycone, which yields CGP 41251 (Fig. 1), abolishes the ability of the parent molecule to translocate nPKCs, whereas a hydroxy group at carbon 7 of the aglycone to furnish UCN-01 decreases, but does not eliminate, it.

The mechanism by which PKC modulators cause nPKC redistribution in the cell is not well understood. It seems unlikely that PKC inhibitory strength or isoenzyme specificity of the inhibitors are mechanistically associated with translocation. Inhibitory potencies of UCN-01, CGP 41251 and the bisindolylmaleimides *in vitro* are similar, and they all inhibit cPKCs more selectively than nPKCs, with very little effect on aPKCs [21, 22]. In contrast, staurosporine does not discriminate between c and nPKC isoenzyme types. Intracellular movement of PKC is thought to involve "receptors for activated C kinase" (RACKs) [23]. It is conceivable that different RACKs bind to different PKC isozymes and that staurosporine, UCN-01, Ro 31-8220, and GF 109203X interact with the RACKs, which specifically transport nPKCs, but not those that mediate translocation of cPKCs. It has been suggested that the nPKC-ε-translocatory activity of staurosporine might be a consequence of its partial PKC-agonistic effect [6, 7] and related to its weak ability to induce tumour promotion [4]. The similar, albeit weaker, effects of UCN-01 and the two bisindolylmaleimides on nPKC localisation solicit the question as to whether or not they share with staurosporine PKC activatory and tumour promotory properties. This hypothesis needs to be investigated, particularly with regard to the potential therapeutic application of some of these agents.

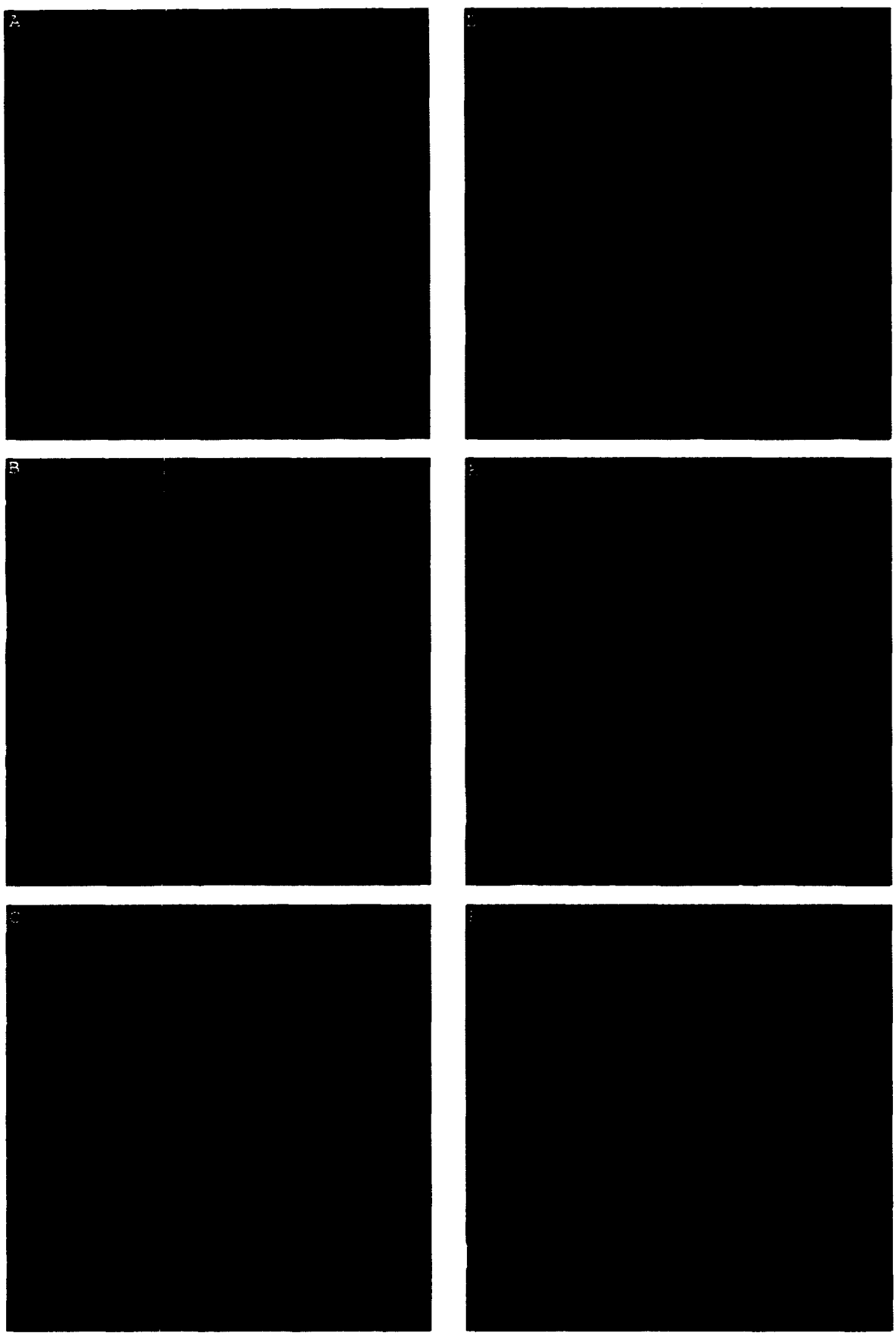

FIG. 3. Intracellular localisation of PKC-θ in A549 cells (A) and A549 cells after exposure for 30 min to TPA (25 nM) (B), staurosporine (C), GF 109203X (D), Ro 31-8220 (E), or UCN-01 (F) (1 μM each). Cells were incubated with or without agent prior to indirect immunostaining with anti-PKC-θ antibody. The secondary antibody was FITC conjugated antimouse IgG, and nuclei were counterstained with PI. Scale bar: 20 μm. For details of staining and confocal microscopy see Materials and Methods.

This work was supported by Grant SP 2233 from the UK Cancer Research Campaign. We thank Dr. T. Tamaoki (Kyowa Hakko Kogyo Co., Tokyo, Japan), Dr. D. Fabbro (Novartis Ciba Geigy, Basle, Switzerland), and Dr. D. Bradshaw (Roche Research Centre, Welwyn Garden City, UK) for samples of UCN-01, CGP 41251, and Ro 31-8220, respectively.

References

1. Nishizuka Y, Intracellular signaling by hydrolysis of phospholipids and activation of PKC. *Science* **258:** 607–614, 1992.
2. Borner C and Fabbro D, PKC and cellular transformation. In: *Protein Kinase C—Current Concepts and Future Perspectives.* (Eds. Lester DS, Epand R), pp. 297–354. Ellis Horwood Ltd, Chichester, 1992.
3. Jaken S, Protein kinase C isozymes and substrates. *Curr Opin Cell Biol* **8:** 168–173, 1996.
4. Yoshizawa S, Fujiki H, Suguri H, Suganuma M, Nakayasu M, Matsushimna R and Sugimura T, Tumor-promoting activity of staurosporine, a protein kinase inhibitor, in mouse skin. *Cancer Res* **50:** 4974–4978, 1990.
5. Yamada S, Hirota K, Chida K and Kuroki T, Inhibition of phorbol ester-caused induction of ornithine decarboxylase and tumor promotion in mouse skin by staurosporine, potent inhibitor of protein kinase C. *Biochem Biophys Res Commun* **157:** 9–15, 1988.
6. Sako T, Tauber AI, Jeng AY, Yuspa SH and Blumberg PM, Contrasting actions of staurosporine, a protein kinase C inhibitor, on human neutrophils and primary mouse epidermal cells. *Cancer Res* **48:** 4646–4650, 1988.
7. Dlugosz AA and Yuspa SH, Staurosporine induces protein kinase-C agonist effects and maturation of normal and neoplastic mouse keratinocytes *in vitro*. *Cancer Res* **51:** 4671–4684, 1991.
8. Kiley SC, Parker PJ, Fabbro D and Jaken S, Selective redistribution of protein kinase C isozymes by thapsigargin and staurosporine. *Carcinogenesis* **13:** 1997–2001, 1992.
9. Tamaoki T and Nakano H, Potent and specific inhibitors of protein kinase C of microbial origin. *Biotechnology* **8:** 732–735, 1990.
10. Meyer T, Regenass U, Fabbro D, Alteri E, Rösel J, Müller M, Caravatti G and Matter A, A derivative of staurosporine (CGP 41251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vitro* antitumour activity. *Int J Cancer* **43:** 851–856, 1989.
11. Akinaga S, Gomi K, Morimoto M, Tamaoki T, and Okabe M, Antitumor activity of UCN-01, a selective inhibitor of protein kinase C, in murine and human tumor models. *Cancer Res* **51:** 4888–4892, 1991.
12. Courage C, Budworth J, Gescher A, Comparison of ability of protein kinase C inhibitors to arrest cell growth and to alter cellular protein kinase C localisation. *Br J Cancer* **71:** 697–704, 1995.
13. Chang JD, Xu Y, Raychowdhury MK and Ware JA, Molecular cloning and expression of a cDNA encoding a novel isoenzyme of protein kinase C (nPKC). *J Biol Chem* **268:** 14208–14214, 1993.
14. Baier G, Baier-Bitterlich G, Meller N, Coggeshall KM, Giampa L, Telford D, Isakov N and Altman A, Expression and biochemical characterisation of human protein kinase C-θ. *Eur J Biochem* **225:** 195–203, 1994.
15. Baier G, Telford D, Giampa L, Coggeshall KM, Baier-Bitterlich G, Isakov N and Altman A, Molecular cloning and characterization of PKC-θ, a novel member of the protein kinase C (PKC) family expressed predominantly in hematopoietic cells. *J Biol Chem* **268:** 4997–5004, 1993.
16. Greif H, Ben-Chaim J, Shimon T, Bechor E, Eldar H and Livneh E, The protein kinase C related PKC-L (η) gene product is localized in the cell nucleus. *Mol Cell Biochem* **12:** 1304–1311, 1992.
17. Stanwell C, Gescher A, Bradshaw TD and Pettit GR, The role of protein kinase C isoenzymes in growth inhibition caused by bryostatin 1 in human A549 lung and MCF-7 breast carcinoma cells. *Int J Cancer* **56:** 585–592, 1994.
18. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
19. Davis PD, Elliott L, Harris W, Hurst SA, Keech E, Kumar H, Lawton G, Nixon JS and Wilkinson SE, Inhibitors of protein kinase C. 2. Substituted bisindolylmaleimides with improved potency and selectivity. *J Med Chem* **35:** 994–1001, 1992.
20. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D and Kirilovsky J, The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266:** 15771–15781, 1991.
21. Wilkinson SE, Parker PJ and Nixon JS, Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem J* **294:** 335–337, 1993.
22. Seynaeve CM, Kazanietz MG, Blumberg PM, Sausville EA and Worland PJ, Differential inhibition of protein kinase C isozymes by UCN-01, a staurosporine analogue. *Mol Pharmacol* **45:** 1207–1214, 1994.
23. Mochly-Rosen D, Khaner H, Lopez J and Smith BL, Intracellular receptors for activated protein kinase C. *J Biol Chem* **266:** 14866–14868, 1991.