The catalytic domain limits the translocation of protein kinase C α in response to increases in Ca²⁺ and diacylglycerol

Arathi RAGHUNATH, Mia LING and Christer LARSSON¹

Department of Laboratory Medicine, Molecular Medicine, Lund University, Entrance 78, 3rd Floor, Malmö University Hospital, 205 02 Malmö, Sweden

Translocation of protein kinase C (PKC) α , β II, δ and ϵ fused to enhanced green fluorescent protein (EGFP) was studied in living neuroblastoma cells by confocal microscopy. Exposure to carbachol elicited transient translocation of PKC α -EGFP and β II-EGFP in most of the cells, PKC δ -EGFP in a few cells and induced sustained translocation of PKC ϵ -EGFP. To monitor levels of Ca²⁺ and diacylglycerol and the translocation of PKC in the same cell, the Ca²⁺-sensitive C2 domain, diacylglycerol-sensitive C1 domains and full-length PKC were fused to red, cyan and yellow fluorescent proteins respectively. PKC α was translocated a few seconds after the C2 domain, which represents an increase in Ca²⁺. This delay was insensitive to removal of the pseudosubstrate in PKC α , but the isolated regulatory domain

translocated simultaneously with the C2 domain. Translocation of PKC ϵ coincided with the increase in diacylglycerol. Ionomycin induced translocation of PKC α and the C2 domain, whereas 1,2-dioctanoylglycerol caused translocation of the C1 domains and PKC ϵ , but not PKC α . Experiments with individual C1 domains showed that treatment with carbachol or phorbol 12,13-dibutyrate elicited translocation of PKC α C1a, PKC ϵ C1a and PKC ϵ C1b, whereas PKC α C1b was largely insensitive to these agents. In contrast with full-length PKC α , the regulatory domain of PKC α and pseudosubstrate-devoid PKC α responded to the carbachol-stimulated increase in diacylglycerol.

Key words: C1 domain, muscarinic, neuroblastoma cell.

INTRODUCTION

Members of the protein kinase C (PKC) family of related serine/threonine kinases play important and diverse roles in cellular regulation. There are at least ten different PKCs, which can be subdivided into classical (PKC α , β I, β II and γ), novel (PKC δ , ϵ , η and θ) and atypical (PKC ι / λ and ζ) isoforms. Classical and novel PKCs are presumably activated after stimulation of receptors coupled with phospholipase C β (G-protein-coupled receptors) or γ (receptor tyrosine kinases). This leads to the formation of 1,2-diacylglycerol (DAG) and increased intracellular Ca²⁺ levels due to release from inositol 1,4,5-trisphosphate-sensitive Ca²⁺ stores. Both these second messengers can activate the classical PKCs, whereas the novel isoforms are sensitive only to DAG [1–5].

Stimulation of receptors coupled with phospholipase C often leads to translocation of different classical and novel PKC isoforms to the plasma membrane or other intracellular sites. Such transfer to the cell membrane depends on increases in Ca²⁺ and DAG, which interact with the C2 and C1 domains respectively and thereby target the PKC molecule to anionic membranes. *In vitro* experiments have shown that the initial step in the association of classical PKCs with the cell membrane is Ca²⁺-mediated binding of the C2 domain to anionic phospholipids, which in turn facilitates the interaction of DAG with the C1 domains [6]. This was supported by the results of a study of living cells in which PKC γ was fused to green fluorescent protein (GFP), which also led to a model proposing that in the inactive cytosolic enzyme, the C2 domain is directly accessible to Ca²⁺, whereas the DAG-binding site is hidden by the catalytic domain [7]. The accessibility of C2 and C1 domains to PKC activators has not yet been elucidated for other isoforms.

It is probable that the PKC isoforms have unique functions that are largely determined by targeting of individual isoforms to

their sites of action [8,9]. All the classical and novel isoforms are sensitive to second messengers that are increased after activation of phospholipase C. It could therefore be expected that these PKC isoforms are activated on stimulation of receptors that are coupled with phospholipase C. However, it has commonly been observed that relatively specific subsets of PKC isoforms are activated after stimulation of such receptors. Thus it would be interesting to understand the mechanisms that determine which PKCs will be translocated under circumstances when phospholipase C is activated.

Stimulation of muscarinic acetylcholine receptors has been shown to influence neuronal cells in a number of ways. Studies of cultured neuroblastoma cells have demonstrated that PKC is involved in several of these effects, including activation of extracellular signal-regulated protein kinase 1/2 [10,11], regulation of gene expression [12–17] and cytoskeletal rearrangements [18]. However, little is known about the identity of the isoforms that are activated by muscarinic receptors or about the temporal and spatial profiles of translocation exhibited by different PKCs on stimulation of muscarinic receptors.

The present study was designed to identify the PKC isoforms that are activated on carbachol stimulation of human neuroblastoma cells and also to pinpoint the domains in the isoforms that determine the translocation pattern. To achieve this objective, all classical and novel PKC isoforms, which are normally expressed in neuroblastoma cells [19,20], were tagged with enhanced GFP (EGFP), and localization of these fusion proteins during exposure to carbachol was monitored by confocal microscopy. This demonstrated specific temporal translocation patterns of the isoforms. To compare the temporal patterns of translocation with increases in the second messengers Ca²⁺ and DAG in individual cells, different fluorescent proteins were fused to the DAG-sensitive C1 domains, Ca²⁺-sensitive C2 domain and full-length PKC. These experiments indicated that PKC α only

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; BAPTA/AM, BAPTA tetrakis(acetoxymethyl ester); DAG, 1,2-diacylglycerol; DOG, 1,2-dioctanoylglycerol; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced GFP; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; RD, regulatory domain.

¹ To whom correspondence should be addressed (e-mail Christer.Larsson@molmed.mas.lu.se).

responded to Ca^{2+} and not to DAG, which led us to investigate the roles of different PKC α domains in determining this response.

EXPERIMENTAL

Plasmids

Cytomegalovirus-promoter-driven expression vectors encoding full-length PKC isoforms and the regulatory domain (RD) of PKC α fused to EGFP have been described previously [20,21]. Vectors encoding other PKC α and PKC ϵ constructs were created by PCR amplification of the desired PKC cDNA using the primers listed in Table 1 and the PKC α or PKC ϵ expression vectors as template. The PCR fragments were digested with *Bgl*II–*Sal*I and ligated into these sites in the pEGFP-N1 vector. All PCR-generated fragments were sequenced to confirm that no mutations had been introduced in the PCR. The various PKC α constructs are illustrated in Figure 1. To generate fusions with enhanced cyan fluorescent protein (ECFP), enhanced yellow fluorescent protein (EYFP) and DsRed, PKC cDNAs were excised from the EGFP vector by using *Bgl*II–*Sal*I and these were inserted into expression vectors containing cDNA for the above-mentioned fluorescent proteins (BD Biosciences Clontech, Palo Alto, CA, U.S.A.).

Cell culture and transfection of cells

SK-N-BE(2) neuroblastoma cells were used in all experiments and they were maintained in minimal essential medium supplemented with 10% foetal calf serum, 100 IU/ml penicillin and

100 $\mu\text{g}/\text{ml}$ streptomycin. For confocal imaging and immunofluorescence experiments, the cells were seeded on glass coverslips in culture dishes at a density of 3.5×10^5 cells/dish. The cells were transfected using Lipofectamine 2000 (Gibco BRL, Basel, Switzerland) according to the manufacturer's instructions.

Confocal microscopy of PKC translocation and intracellular Ca^{2+} fluctuations

Cells were examined by confocal microscopy on a day after transfection. In some experiments, the cells were labelled with 2 μM Fura Red (Molecular Probes, Leiden, The Netherlands) for 40 min or with 20 μM BAPTA/AM [bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) tetrakis(acetoxy-methyl ester); Sigma] for 30 min before the analysis. The coverslips were washed twice with buffer H [20 mM Hepes/137 mM NaCl/3.7 mM KCl/1.2 mM MgSO_4 /2.2 mM KH_2PO_4 /1.6 mM CaCl_2 /10 mM glucose (pH 7.4)] and mounted on the heated stage of a Nikon microscope. The cells were incubated in buffer H and examined using a $\times 60$ objective (numerical aperture 1.4) and a Bio-Rad Radiance 2000 confocal system with excitation wavelengths at 488 nm (EGFP) and 543 nm (Fura Red) and emission filters 515HQ30 (EGFP) and 600LP (Fura Red). Images were captured every 5 s and five images were taken before adding one of the following (all obtained from Sigma): 1 mM carbachol, 1 μM ionomycin, 100 μM 1,2-dioctanoylglycerol (DOG) or 1 μM phorbol 12,13-dibutyrate (PDBu). Thereafter, LaserPix software was used to analyse fluorescence intensities in the cytosol. Since the cells changed shape during experiments, an area of interest for intensity analysis was carefully selected to ensure that it actually represented the cytosol throughout the experiment. The average of the intensities of the five images before addition of stimulus was used as a baseline value and set at 100%. Non-negligible photobleaching of the fluorescent proteins occurred during the experiments. To compensate for this effect, the value for each image was adjusted using the quotient obtained by dividing the total fluorescence intensity in a large area (frequently encompassing several cells and found to display a linear decrease) by the intensity in the area at the beginning of the experiment.

Confocal microscopy for detection of ECFP, EYFP and DsRed in the same cell

To avoid bleed-through, the fluorochromes were imaged sequentially using excitation wavelengths of 457, 514 and 543 nm and the emission filters HQ485/30, HQ545/40 and HQ600/50 for ECFP, EYFP and DsRed respectively. Images (256 $\times$ 256 pixels) were acquired at a scanning speed of 750 lines/s, and with the software we used in the Radiance confocal system, this resulted in a time interval of approx. 1.3 s between the images of the different fluorochromes. Images were obtained every 5 s and quantification and compensation for bleaching were done as described for the EGFP measurements. The laser strobe function was utilized in experiments in which images were recorded every second.

Immunofluorescence

Cells were fixed for 4 min with 4% (w/v) paraformaldehyde in PBS and then incubated for 30 min with 0.3% (v/v) Triton X-100 and 5% goat serum in PBS. Thereafter, the cells were incubated with primary polyclonal antibodies towards PKC α (1:500), βII , δ or ϵ (all 1:100) or a monoclonal antibody to PKC βI (1:100) for 1 h in PBS (all antibodies from Santa Cruz Biotechnology, Heidelberg, Germany) and then washed three times

Table 1 Primers used to generate PKC constructs

Construct	Primers
PKC α C1	Forward: GGAAGATCTGGACCATGGCTGACGTTTTC Reverse: CGCGTCGACTCAGTGTGATCCATTCCGCA
PKC α C2	Forward: GCGAGATCTGGACCATGGACCTGAGAAGAGGGGCGGATT Reverse: AACGTCGACGACGGGCAAGTTTGGCTTT
PKC α C1a	Forward: GCGAGATCTGGACCATGGAGGTGAAGGACCACAAATTC Reverse: GCGGTCGACTGGATTTTGAACCTTGCTTGCT
PKC α C1b	Forward: GCGAGATCTGGACCATGGACCCAGGAGCAAGCACAAG Reverse: GCGGTCGACTTTTCATCAGCAACCTCAGCCTT
PKC α FL(ΔelPS)	Forward: GCGAGATCTGGACCATGGAGGTGAAGGACCACAAATTC Reverse: CGCGTCGACCATCTGCACTCTGTAAGATG
PKC α C1a	Forward: GCGAGATCTGGACCATGAGGTCAACGCGCCACAAGTTC Reverse: GCGGTCGACATGTTGACGCTGAACCGCTGGGA
PKC α C1b	Forward: GCGAGATCTGGACCATGGTCAACATGCCCAACAAGTTC Reverse: GCGGTCGACATTTGTCTGGGGTAACGCCAG

Figure 1 Composition of the different PKC α constructs used in the present study

The domains that comprise the different PKC α constructs are shown. P, pseudosubstrate; CD, catalytic domain.

with PBS and exposed to Alexa Fluor 488-labelled goat-anti-rabbit antibodies (1:400; Molecular Probes) for 1 h. The cells were analysed by confocal microscopy as described above for the EGFP-tagged PKC isoforms.

RESULTS

Carbachol induces translocation of endogenous and EGFP-tagged PKC isoforms

Initially, to evaluate the usefulness of EGFP-tagged PKC isoforms as indicators of the behaviour of endogenous PKCs, we compared overexpressed PKC α , β I, β II, δ and ϵ fused to EGFP with the corresponding endogenous isoforms with regard to their subcellular distribution (Figure 2). PKC α , β II and ϵ were predominantly found in the cytoplasm, where there was perinuclear enrichment of PKC ϵ and more uniform distribution of PKC α and β II. We also discerned some PKC ϵ immunoreactivity in the nucleus, and the intensity of this staining in relation to the intensity in the cytosol varied slightly between experiments. PKC δ was also found at different levels both in the nucleus and the cytoplasm. The endogenous PKC α , β II, δ and ϵ isoforms showed distribution patterns that were essentially identical with those of their corresponding EGFP fusions. However, endogenous PKC β I was mainly found within the nucleus, whereas the PKC β I-EGFP fusion protein occurred exclusively outside the nucleus. Thus the distribution patterns of EGFP fusions of the α , β II, δ and ϵ isoforms, but not PKC β I, seem to represent localization of the endogenous proteins.

Stimulation of muscarinic receptors induces activation of phospholipase C and also causes numerous PKC-mediated effects in neuroblastoma cells. We therefore performed confocal microscopy to ascertain whether the stable acetylcholine analogue carbachol could induce translocation of the overexpressed PKC-EGFP isoforms in living SK-N-BE(2) cells (Figure 3). We examined PKC α , β II, δ and ϵ , for which we had found similar or identical localization patterns for the endogenous and EGFP-fused proteins. Carbachol caused a rapid but transient translocation of PKC α and β II to the cell membrane in most of the cells. The temporal pattern was consistent, comprising maximal translocation of PKC-EGFP within 1.5 min followed by a return to the localization seen in untreated cells. However, PKC δ -EGFP was seldom redistributed after exposure to carbachol. In the few cells in which PKC δ was translocated, we observed a transient effect similar to that seen with PKC α and β II. For PKC ϵ , stimulation with carbachol induced a unique temporal pattern of sustained translocation in most of the cells investigated.

To estimate the time course of the translocations, the intensity of EGFP fluorescence was quantified in a selected area of the cytosol in responding cells (Figure 3B). Owing to changes in the shape of the cells over time, the fluorescence intensity of the plasma membrane could not be quantified and the area of the cytosol that could be monitored was limited. Consequently, for each quantification we selected an area of the cytosol that did not contain plasma membrane or nuclear components at any time during the experiment. These analyses confirmed that translocation was sustained for PKC ϵ but was transient for PKC α and β II and also for PKC δ , in the few cells in which this isoform was translocated.

To confirm that the translocation of the PKC-EGFP fusion proteins was representative of localization of the corresponding endogenous isoforms, we stimulated SK-N-BE(2) cells with carbachol and then analysed, by immunofluorescence methodology, the subcellular localization of endogenous PKC α and ϵ (Figure 4), isoforms that exemplify two different temporal patterns of translocation. We found that, after exposure to

Figure 2 Subcellular localization of PKC-EGFP fusion proteins and endogenous PKC isoforms in SK-N-BE(2) cells

The cells were transfected with expression vectors encoding the PKC isoforms α (A), β I (C), β II (E), δ (G) and ϵ (I) fused to EGFP, and 16 h later, the cells were fixed and examined by confocal microscopy. Non-transfected SK-N-BE(2) cells were analysed with primary antibodies specific for PKC α (B), β I (D), β II (F), δ (H) and ϵ (J) and secondary antibodies conjugated with Alexa Fluor 488. The cells were thereafter examined by confocal microscopy.

carbachol, the native proteins displayed localization similar to that of their EGFP fusions. There was a marked transfer of endogenous PKC α to the plasma membrane after 30 s of carbachol treatment, and this was largely reversed after 2 min, indicating the transient nature of the response. In contrast, exposure to carbachol led to sustained movement of PKC ϵ to the plasma membrane.

Figure 3 For legend, see facing page

Figure 4 Subcellular localization of endogenous PKC α and ϵ after stimulation with carbachol

SK-N-BE(2) cells were treated with 1 mM carbachol after which immunofluorescence methodology was used to visualize endogenous PKC α (A–C) and ϵ (D, E). The images show unstimulated cells (A, D) and cells exposed to carbachol for 30 s (B), 2 min (C) and 10 min (E).

Carbachol-stimulated PKC translocation and increases in Ca²⁺ and DAG visualized in the same cell

The experiments with PKC-EGFP fusions revealed two important features. First, all cells did not display PKC translocation in response to carbachol. Secondly, there are two patterns of PKC translocation, one entailing a transient redistribution, exemplified by PKC α and the other involving sustained relocation as detected for PKC ϵ . It is probable that the translocation of PKC isoforms is caused by increases in levels of intracellular Ca²⁺ and DAG, which are caused by the activation of phospholipase C. Lack of translocation of PKC could be due either to an inherent incapacity of the PKC molecule to be translocated in that particular cell or to the fact that phospholipase C in the cell is not activated in response to carbachol. Monitoring Ca²⁺ and DAG levels would enable temporal correlation of PKC translocation to increases in concentrations of these second messengers, and it would also constitute an internal indicator of whether or not carbachol has increased the levels of Ca²⁺ and DAG in the cell being studied.

It has been demonstrated previously [7,22] that the C2 and C1 domains of PKC γ respond to Ca²⁺ and DAG respectively by translocating to the plasma membrane. Therefore, to monitor the levels of these messengers, we constructed expression vectors encoding the Ca²⁺-sensitive C2 domain and the DAG-binding C1 domains of PKC α . Both cDNAs were fused to EGFP cDNA,

Figure 5 Translocation of the PKC α C2 domain is correlated with elevated levels of intracellular Ca²⁺

SK-N-BE(2) cells transfected with an expression vector encoding the C2 domain of PKC α fused to EGFP were incubated for 40 min with the Ca²⁺-sensitive dye Fura Red. Thereafter, the cells were stimulated with 1 mM carbachol and the fluorescence of EGFP and Fura Red was monitored by confocal microscopy. The diagram shows the fluorescence intensity of both EGFP and the Ca²⁺ indicator Fura Red in the cytosol of the same cell before and after addition of carbachol (indicated by arrows). Decreased fluorescence of Fura Red represents an increase in Ca²⁺.

and to allow simultaneous monitoring of these domains in the same cell, we fused PKC α C1 cDNA to ECFP cDNA and PKC α C2 cDNA to DsRed cDNA. To confirm that a translocation of the C2 domain really represents an increase in Ca²⁺, we transfected SK-N-BE(2) cells with the vector encoding PKC α C2-EGFP and also loaded them with Fura Red to be able to determine levels of intracellular Ca²⁺. The cells were stimulated with carbachol and the fluorescence intensity of EGFP and Fura Red in the cytosol was monitored by confocal microscopy and quantified (Figure 5). Carbachol induced a rapid increase in intracellular Ca²⁺ levels, which gradually levelled off. This pattern was consistent for all experiments with carbachol. In contrast with several other cell types [7,23–26], no oscillations of either Ca²⁺ levels or of PKC translocations could be observed. We found that translocation of the C2 domain to the plasma membrane paralleled the increase in intracellular Ca²⁺, suggesting that it is indeed a good indicator of Ca²⁺ levels within cells. Consequently, in subsequent experiments, we used the C2 domain as an indicator of cellular responsiveness to carbachol, and only cells that displayed translocation of the C2 domain on treatment with carbachol were analysed.

To monitor PKC α C1-ECFP, PKC α -EYFP and PKC α C2-DsRed in the same cell, we co-transfected SK-N-BE(2) cells with vectors encoding these fusion proteins. On the following day, we treated them with carbachol and performed confocal microscopy to monitor the intracellular distribution of the fusion proteins. Carbachol induced transient translocation of PKC α C2-DsRed to the plasma membrane and sustained redistribution of

Figure 3 Carbachol-stimulated translocation of PKC isoforms fused to EGFP

SK-N-BE(2) cells transfected with expression vectors encoding PKC α , β II, δ and ϵ fused to EGFP were stimulated with 1 mM carbachol, and the localization of the fusion proteins was monitored every 5 s for 7.5 min by confocal microscopy. (A) Images of individual cells expressing either PKC α , β II, δ or ϵ at different time points after addition of carbachol. (B) Fluorescence intensity of EGFP in the cytosol of responding cells was quantified for the different PKC isoforms. The data represent means $\pm$ S.E.M. for 6–9 determinations from 2–3 separate experiments and are expressed as percentage of the levels in unstimulated cells. Time (min) is given on the x-axis, and arrows indicate addition of carbachol.

Figure 6 Visualization of carbachol-stimulated translocation of PKC α C1-ECFP, PKC α C2-DsRed and either PKC α - or PKC ϵ -EYFP in the same cell

SK-N-BE(2) cells transfected with vectors encoding PKC α C1-ECFP, PKC α -EYFP and PKC α C2-DsRed were stimulated with carbachol, and the subcellular localization of the different constructs was visualized by confocal microscopy. **(A)** Images of representative cells showing the subcellular localization of the fusion proteins at different time points after addition of carbachol. **(B)** Traces of the fluorescence intensity of PKC α C1-ECFP, PKC α -EYFP and PKC α C2-DsRed in an area of the cytosol in individual cells that responded to carbachol. Results are expressed as percentage of the levels before stimulation (average of four consecutive images) and are given as means $\pm$ S.E.M. for ten separate cells. **(C)** Traces of PKC α C1-ECFP, PKC ϵ -EYFP and PKC α C2-DsRed in individual responding cells quantified as in **(B)** ($n = 4$). **(B, C)** The blue lines represent PKC α C1-ECFP, the green PKC α -EYFP or PKC ϵ -EYFP and the red line represents PKC α C2-DsRed. The arrows indicate addition of carbachol.

PKC α C1-ECFP (Figure 6A). The latter reaction is similar to the response to carbachol shown by cells expressing PKC α C1-EGFP alone (results not shown), which agrees with the sustained carbachol-induced accumulation of DAG in neuroblastoma cells. This demonstrates that the capacity of the C1 and C2 domains to respond to carbachol was not influenced by the fact that three different proteins fused to different fluorescent proteins were overexpressed in the same cell. Furthermore, as we had seen with PKC α -EGFP, PKC α -EYFP transiently translocated to the membrane in most of the cells. However, movement of full-length PKC α and the isolated PKC α C1 domains was somewhat delayed when compared with the redistribution of the PKC α C2 domain. At some early time points, there was distinct translocation of PKC α C2 but no sign of movement of the full-length protein.

The above-mentioned findings are supported by our quantification of the fluorescence intensities of the different fusion proteins in the cytosol (Figure 6B). Calculating the time point at which 50% of maximal translocation had occurred, we obtained the following values: 15 ± 2.6 s for the PKC α C2 domain, 25 ± 3.9 s for full-length PKC α and 32.5 ± 5.2 s for the PKC α C1 domains.

We used the same approach to delineate the involvement of DAG and Ca²⁺ in carbachol-stimulated translocation of PKC ϵ . SK-N-BE(2) cells were transfected with the vectors encoding PKC α C1-ECFP and PKC α C2-DsRed together with a vector encoding PKC ϵ fused to EYFP. The cells were exposed to carbachol, and the localization of the fusion proteins was monitored by confocal microscopy (Figure 6C). The PKC α C2 domain displayed the rapid transient translocation described

Figure 7 The importance of Ca^{2+} in regulating the translocation of PKC α C1-ECFP, PKC α -EYFP and PKC α C2-DsRed

SK-N-BE(2) cells transfected with vectors encoding PKC α C1-ECFP, PKC α -EYFP and PKC α C2-DsRed were treated with 1 μM ionomycin (A–C) or preloaded with 20 μM BAPTA/AM before stimulation with 1 mM carbachol (D). The subcellular localization of the different fusion proteins was subsequently visualized by confocal microscopy. Fluorescence intensities of the three fusion proteins in the cytosol of cells displaying sustained translocation of the C2 domain were quantified, and the results are presented in (A) as means $\pm$ S.E.M. for five different cells. A few

Figure 8 Effect of DOG on translocation of PKC α C1-ECFP, PKC α -EYFP and PKC α C2-DsRed

SK-N-BE(2) cells transfected with vectors encoding PKC α C1-ECFP, PKC α -EYFP and PKC α C2-DsRed were stimulated with 100 μM DOG, and the subcellular localization of the different fusion proteins was visualized by confocal microscopy. The images show localization of the three fusion proteins in the same cells at different time points after the addition of 100 μM DOG.

above, whereas the response of the PKC α C1 domains was slower. The temporal profile of the movement of PKC ϵ was identical with that of the PKC α C1 domains.

Effects of Ca^{2+} and DOG on translocation of PKC α and PKC ϵ

To investigate further the roles of the second messengers DAG and Ca^{2+} in regulating localization of PKC α , we used the methodology described above to study translocation of the C1 and C2 domains and full-length PKC α in SK-N-BE(2) cells treated with ionomycin or DOG. Ionomycin caused rapid and sustained translocation of both the PKC α C2 domain and the full-length PKC α in most cells (Figure 7A). In a few cells, the movement of the PKC α C2 domain was transient (Figures 7B and 7C) and was accompanied by either transient or no translocation of full-length PKC α . Treatment with DOG induced rapid translocation of the PKC α C1 domains that reached a maximum within 60 s (Figure 8), but caused no movement of either the PKC α C2 domain or full-length PKC α . Exposure to DOG also led to profound changes in the shape of the cells, which made quantification impossible. A concomitant exposure to DOG and ionomycin led to sustained translocations of both the isolated C1 and C2 domains as well as of full-length PKC α (results not shown), reflecting a combined effect of the agents. In contrast with the insensitivity of PKC α , a concentration of DOG as low as 3 μM was sufficient to cause translocation of PKC ϵ in some cells (results not shown).

The importance of Ca^{2+} for carbachol-stimulated PKC α translocation was investigated by loading the cells with BAPTA

cells showed only transient translocation of the C2 domain, and the traces in (B, C) depict the cytosolic intensities of the three fusion proteins in two such cells. The response to carbachol in a cell preloaded with BAPTA/AM is shown in (D). Arrows indicate the addition of ionomycin (A–C) or carbachol (D). Narrow black lines with solid squares represent PKC α C1-ECFP, thick black lines without symbols represent PKC α -EYFP and grey lines represent PKC α C2-DsRed.

Figure 9 PDBu-induced translocation of the C1 domains of PKC α and PKC ϵ

SK-N-BE(2) cells were transfected with vectors encoding EGFP fusion of the C1a or C1b domains of PKC α (α C1a and α C1b) or PKC ϵ (ϵ C1a and ϵ C1b). On the following day, the cells were treated with 1 μ M PDBu and localization of the fusion proteins was monitored by confocal microscopy. Confocal images show localization of the C1 domains before and after treatment with PDBu for 10 min.

before stimulation (Figure 7D). Carbachol-stimulated translocation of the PKC α C2 domain and of full-length PKC α were abolished in BAPTA-treated cells, whereas the PKC α C1 domains relocated to the plasma membrane under these conditions.

Translocation of individual C1 domains of PKC α and PKC ϵ

PKC α did not respond to either endogenous DAG generated by treatment with carbachol or to exogenously added DOG, whereas PKC ϵ reacted to both these stimuli. These results may be due to different affinities of the C1 domains for DAG. To investigate the sensitivity of the C1 domains, we constructed expression vectors encoding the different PKC α and PKC ϵ C1 domains fused to EGFP and exposed neuroblastoma cells expressing these domains to the known C1 agonist PDBu (Figure 9). Treatment with 1 μ M PDBu caused PKC α C1a, PKC ϵ C1a

Figure 10 Carbachol-induced translocation of the C1 domains of PKC α and PKC ϵ

SK-N-BE(2) cells were co-transfected with vectors encoding EGFP fusions of the C1a or C1b domains of PKC α (α C1a and α C1b) or PKC ϵ (ϵ C1a and ϵ C1b), together with a vector encoding PKC α C2-DsRed. On the following day, the cells were stimulated with 1 mM carbachol and localization of the fusion proteins was monitored by confocal microscopy. (A) Confocal images showing localization of the C1 domains before and after treatment with carbachol for 2 min. (B) Percentage of cells displaying carbachol-induced translocation of a C1 domain. Only cells in which PKC α C2 was translocated were included in the quantification; this was done to exclude cells that did not respond to carbachol in terms of activation of the phospholipase C pathway.

Figure 11 Carbachol induces sustained translocation of PKC α RD and PKC α lacking pseudosubstrate

SK-N-BE(2) cells were co-transfected with vectors encoding EGFP fusions of PKC α RD (A) or PKC α devoid of pseudosubstrate [PKC α (delPS)] (B) together with a vector encoding PKC α C2-DsRed. The cells were exposed to carbachol and translocation of the fusion proteins was examined by confocal microscopy. The panels show localization of PKC α C2-DsRed and the respective EGFP fusion in the same cells at indicated time points after addition of carbachol.

and PKC ϵ C1b to translocate to the plasma membrane, although a substantial amount of the EGFP fusion proteins remained in the cytosol, and this was most evident for PKC ϵ C1b-EGFP.

Figure 12 Analysis of the onset of carbachol-stimulated translocation of PKC α , PKC α RD and pseudosubstrate-devoid PKC α

SK-N-BE(2) cells were co-transfected with vectors encoding EGFP-tagged PKC α (A), PKC α RD (B) and PKC α devoid of pseudosubstrate, [PKC α (delPS)] (C) together with a vector encoding PKC α C2-DsRed. The cells were stimulated with carbachol and the laser strobe function of the software was used to acquire simultaneous confocal microscopic images every second. Quantification of the fluorescence intensity of PKC α C2-DsRed in the cytosol revealed that the time elapsing between addition of carbachol and the onset of translocation differed between individual cells. Therefore the responses were temporally normalized to the response of the C2 domain by analysing values of 10 s before and 30 s after initiation of the translocation of the C2 domain. The data represent means $\pm$ S.E.M. ($n = 4-6$).

In contrast, the PKC α C1b domain responded only in 3 out of 5 experiments, and in these cases (exemplified in Figure 9), clearance of nuclear PKC α C1b-EGFP was the sole effect

observed. The C1 domain might have moved to the nuclear membrane, but this was difficult to discern clearly. There was no translocation of PKC α C1b from the cytosol to the plasma membrane.

In subsequent experiments, we investigated whether the C1 domains also differ in their response to carbachol (Figure 10). To ensure that all the cells we examined were responsive to carbachol, we co-expressed PKC α C2-DsRed with the C1 domains and monitored the translocation of this protein as an indicator of sensitivity to carbachol. The profile of the responsiveness of the different C1 domains resembled, but was not as prominent as, the corresponding profile of sensitivity to PDBu. PKC α C1a, PKC ϵ C1a and PKC ϵ C1b domains reacted to carbachol by translocating to the plasma membrane in roughly half of the cells in which the PKC α C2 domain was translocated. The C1 domains returned to the cytosol during carbachol treatment in some experiments, whereas membrane localization persisted in others. The C1b domain of PKC α invariably failed to respond to carbachol stimulation.

Removal of the pseudosubstrate or the catalytic domain makes PKC α responsive to DAG

Our results showing that both C1 domains of PKC ϵ , but only the C1a domain of PKC α , responded to carbachol and PDBu might explain the differences in sensitivity to DAG shown by PKC α and ϵ . We used two different approaches to study whether domains other than the C1 domains may compromise the DAG responsiveness of PKC α . To determine whether the presence of the C2 domain has a negative influence on the effect of DAG, we transfected SK-N-BE(2) cells with a vector encoding the entire RD of PKC α and subsequently exposed them to carbachol (Figure 11A). We observed that the RD translocated to the plasma membrane and did not return to the cytosol, which is in contrast with the pattern of movement exhibited by full-length PKC α , i.e. movement to the plasma membrane and subsequent return to the cytosol once the Ca²⁺ levels decrease. Thus the C2 domain does not block the effect of DAG on the PKC α C1 domains. We therefore hypothesized that the lack of the DAG effect on full-length PKC α is due to the presence of the catalytic domain. It is assumed that the catalytic domain in the inactive PKC molecule is bound to the pseudosubstrate. To investigate whether annulling this binding could render PKC α responsive to DAG, we created a construct that generated PKC α devoid of the pseudosubstrate. Exposure to carbachol caused sustained translocation of this protein to the plasma membrane (Figure 11B). Furthermore, both the PKC α RD and the pseudosubstrate-devoid construct responded to 100 μ M DOG by translocating to the plasma membrane (results not shown), whereas the full-length PKC α was insensitive to DOG, as shown previously (Figure 8).

The isolated C2 domain and PKC α RD respond simultaneously to carbachol stimulation

Another feature of the carbachol-stimulated PKC α translocation was the time lag between translocation of the C2 domain and the full-length protein. To analyse further this delay, we performed experiments in which images were captured every second instead of every 5 s as shown in Figure 6. To study the involvement of other domains in this time lag, we also investigated the temporal pattern of translocation of the entire RD of PKC α and of full-length, pseudosubstrate-devoid PKC α . In line with the results presented in Figure 6, we found that the translocation of full-length PKC α was delayed several seconds compared with the C2

domain (Figure 12A). However, the onset of the translocation occurred simultaneously for the RD and the C2 domain (Figure 12B), whereas the pseudosubstrate-devoid PKC α responded in a manner similar to full-length PKC α (Figure 12C), i.e. the protein translocated a few seconds after the C2 domain.

DISCUSSION

The emergence of GFP has made it possible to visualize signal-transduction pathways in live cells. This is accomplished by monitoring the movement of both GFP-tagged signalling proteins and GFP-tagged domains that react to specific second messengers. The translocation patterns of PKC isoforms fused to GFP variants have been studied extensively in work focused on the following: PKC α in BHK, GH3B6 and vascular smooth muscle cells [27–29]; PKC β II in HEK-293 cells [30]; PKC γ in COS, NG108-15 and rat basophilic leukaemia 2H3 cells [7,31,32]; PKC δ in COS and Chinese-hamster ovary cells [33,34]; and PKC ϵ in COS cells [32]. In several of these investigations, *in vitro* experiments showed that PKC-GFP fusion proteins had enzymic properties similar to those of the free PKC molecules, indicating that fusion to GFP does not compromise the function of PKC. In our comparison of the intracellular distribution of EGFP-fused and endogenous PKCs, only PKC β I-EGFP exhibited aberrant localization, suggesting that fusion to EGFP disturbed the endogenous role of this isoform, hence we did not use PKC β I-EGFP in our study. The EGFP fusions of the other classical and novel isoforms that are expressed in neuroblastoma cells [19] displayed distribution patterns similar to those of the endogenous isoforms. Furthermore, considering the two isoforms we examined in great detail PKC α and ϵ , the EGFP-fused and endogenous molecules showed equivalent patterns of distribution in SK-N-BE(2) cells treated with carbachol. Thus it is probable that the localization of EGFP fusions of these isoforms represents the localization of corresponding endogenous proteins in these cells.

Variants of GFP with different spectral properties, as well as novel fluorescent proteins such as DsRed, have been made available in recent years, which has made it possible to monitor several proteins in the same cell. We took advantage of this methodology and generated fusions comprising the DAG-sensitive PKC α C1-ECFP, the Ca²⁺-sensitive PKC α C2-DsRed and full-length PKC isoforms fused to EYFP. This allowed us to monitor levels of Ca²⁺ and DAG and track the localization of holo-PKC, three important components of the phospholipase C transduction pathway, in the same cell.

Exposure to carbachol led to rapid translocation of the classical PKC α and β II isoforms in the SK-N-BE(2) neuroblastoma cells, and this was probably caused by an increase in intracellular levels of Ca²⁺, which has been shown to cause translocation of these two PKCs [23,29]. This conclusion is supported by our findings that (1) treatment with ionomycin led to translocation of both the isolated Ca²⁺-sensitive C2 domain and the full-length PKC α , (2) these two proteins displayed similar temporal profiles of carbachol-induced translocation and (3) preloading with BAPTA abolished the carbachol-stimulated translocation of full-length PKC α .

In our experiments, the C2 and PKC α membrane localization was generally sustained after ionomycin exposure, which contradicts results from parotid cells in which the ionomycin-induced PKC α translocation was transient [25]. This was attributed to autophosphorylation of PKC α , which would presumably cause a detachment from the plasma membrane. In the present study, the persistence of the PKC α translocation was enhanced by removal of the pseudosubstrate. This protein is

probably constitutively active and thus displays a high degree of autophosphorylation, suggesting that autophosphorylation does not lead to PKC detachment from the plasma membrane in neuroblastoma cells.

In contrast with the ionomycin effect, translocation of both the C2 domain and full-length PKC α was transient after carbachol treatment, despite the fact that the Ca²⁺ levels did not completely return to unstimulated conditions. Since no oscillations could be observed, it is conceivable that the difference in sustainability is due to either the localization or the amplitude of the Ca²⁺ response. Relocation of the C2 domain to cytosol temporally correlated with the initiation of the gradual decrease in Ca²⁺ levels. We therefore speculate that the transient nature of the carbachol-stimulated translocation depends on the decrease in Ca²⁺ levels after the peak, suggesting that the translocation of the C2 domain depends on the amplitude of the Ca²⁺ response.

The carbachol-stimulated onset of full-length PKC α translocation was somewhat delayed compared with that of the C2 domain and was also temporally similar to the movement of the DAG-sensitive C1 domains. Such a time lag between an increase in Ca²⁺ and PKC translocation has been observed for both the α [27] and β II [23] isoforms and is presumably not due to slow diffusion, since PKC α has also been shown to translocate half-maximally within 1 s of stimulation of certain receptors [24]. Thus the time it takes for PKC α to translocate after an increase in intracellular Ca²⁺ varies between cell types and there are also cases, exemplified by solitary GH3B6 cells [28], in which an increase in Ca²⁺ does not affect the localization of PKC α . Therefore translocation of PKC α is probably governed not solely by a Ca²⁺-mediated interaction with the membrane but by other components as well.

We found that, after removal of the catalytic domain of PKC α , the isolated RD responded as quickly as the C2 domain, whereas deletion of the pseudosubstrate in full-length PKC α did not influence the time lag. This suggests that the presence of the catalytic domain, whether bound to the pseudosubstrate or not, limits the sensitivity to Ca²⁺. There are several possible explanations for the above-mentioned results. First, differentially expressed proteins may interact with the catalytic domain and influence the translocation pattern of the enzyme. Moreover, the phosphorylation status of residues in the catalytic domain of the PKC molecule, which has been shown to affect the responsiveness of PKC β II to Ca²⁺ [35], could differ between cells. Another feasible model is that the mere presence of the catalytic domain lowers the sensitivity to Ca²⁺, making a smaller increase in Ca²⁺, such as that induced by carbachol, ineffective in causing a translocation of PKC α , unless the levels of DAG are concomitantly elevated. This model is in line with our finding that in some cells in which ionomycin elicited a transient translocation of the C2 domain, the Ca²⁺ ionophore had no effect on full-length PKC α .

We also found that neither exogenously applied DOG nor endogenously produced DAG could cause translocation of PKC α , although both these treatments did lead to translocation of PKC ϵ . In the light of the varying sensitivity of the C1 domains to agonists, we investigated the response of the individual C1 domains of PKC α and ϵ to PDBu and carbachol. It has been reported that the C1b domain, but not the C1a domain, of PKC α binds phorbol esters [36] and that both C1 domains are of equal importance for phorbol ester-mediated translocation of this isoform [37]. However, more detailed *in vitro* studies of the roles played by the different C1 domains in regulating PKC α demonstrated that only the C1a domain has the capacity to penetrate the membrane and bind DAG [38,39]. Our results corroborate with those of previous studies and also demonstrate that, even

intracellularly, the C1a domain of PKC α is the primary target of DAG. This is analogous to the sensitivity of the C1 domains of PKC γ [22], where only the C1a domain responded to receptor-stimulated generation of DAG. On the other hand, both C1 domains of PKC ϵ responded to PDBu and carbachol, which is in agreement with recently reported results indicating that either of the two C1 domains could mediate phorbol ester-induced translocation of PKC ϵ [40]. This difference between the C1 domains of PKC α and ϵ may explain why only PKC ϵ translocated in response to an increase in DAG in our experiments.

It has been suggested that the capacity of the C1a domain, in the inactive PKC α molecule, to penetrate the membrane is limited because it is tethered to the C2 domain [39,41]. This interaction is presumably disrupted by the binding of Ca²⁺/phosphatidylserine to the C2 domain. This may be the reason why, in the present study, PKC α returned to the cytosol at the same time as the levels of Ca²⁺ decreased, despite the sustained increase in DAG. However, we found that removal of either the pseudosubstrate or the entire catalytic domain in PKC α generated proteins containing both the C1 and C2 domains, which responded to an increase in DAG. Thus a putative C1–C2 interaction that blocks the C1a domain seems to depend on an interaction of the catalytic domain with the pseudosubstrate, and in the absence of this binding, the C1a domain is apparently available for interaction with DAG. This situation resembles that described for PKC γ [7], where the C1 domains are supposedly hidden by the catalytic domain in the inactive enzyme, and on interaction of Ca²⁺ with the C2 domain, the C1 domain is exposed and can bind DAG. If this model is correct, our observation that PKC ϵ is responsive to DAG suggests that this isoform, in contrast with classical PKCs, has C1 domains that can be readily accessed by DAG even in the inactive PKC enzyme.

Thus the catalytic domain has a negative influence on the effects of both Ca²⁺ and DAG on PKC α . Removal of the pseudosubstrate and consequently release of the pseudosubstrate-binding of the catalytic site is sufficient to restore the DAG sensitivity. This is in contrast with the response to Ca²⁺, for which we found it necessary to remove the catalytic domain to obtain the same sensitivity exhibited by the isolated C2 domain.

Although PKC δ and ϵ are both novel isoforms, they responded differently to the stimuli we tested. PKC δ was translocated in only a minority of the cells, and the pattern was reminiscent of that exhibited by the classical isoforms. The lack of sustained translocation of PKC δ can be explained by the fact that it is much less responsive to DOG than PKC ϵ (results not shown). Although contradicting the similar sensitivity of the two isoforms to DOG *in vitro* [42], our results also highlight an interesting difference between novel isoforms, which may allow isoform-specific activation upon stimulation of receptors that generate an increase in the levels of DAG. At present, we do not know what mediates the carbachol-stimulated translocation of PKC δ that we observed in a few cells, although this may involve any of a large variety of mechanisms that have been shown to regulate PKC δ [34,43–45].

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