Selective loss of substrate recognition induced by the tumour-associated D294G point mutation in protein kinase Cα

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The tumour-associated D294G mutant of protein kinase Cα (PKCα) was recently shown not to be translocated to the plasma membrane on stimulation with PMA, in contrast with the wild-type enzyme. Using recombinant wild-type and mutant PKCα, we establish here that, although the PKCα intrinsic lipid-dependent catalytic activity remains unaltered by the D294G mutation, the mutant enzyme exhibits a selective loss of substrate recognition. Indeed, whereas the mutant enzyme is still able to phosphorylate histone IIIS with comparable efficiency to that of the wild-type enzyme, it exhibits a lack of kinase activity towards the previously cloned 35F and 35H substrates for PKC. Overlay experiments demonstrate that this selective loss of kinase activity is correlated with a decrease in binding of D294G PKCα to the 35F and 35H proteins compared with that of the wild-type enzyme. Because the 35H and 35F proteins are predicted to be PKCα-anchoring proteins, these findings suggest a selective loss of PKCα–protein interactions that might fail to stabilize the location of the PKCα mutant at the plasma membrane.

INTRODUCTION

Protein kinase C (PKC) is a family of 11 Ser/Thr-specific phospholipid-dependent protein kinases classified into classical (α, β1, β2 and γ), novel [δ, ϵ, η (L), θ and μ] and atypical [ζ and $\iota(\lambda)$] PKCs according to their Ca^{2+} and diacylglycerol requirements for activation [1–4]. Because PKC is the target for tumour promoters, such as phorbol esters [5], several investigations have analysed the involvement of these enzymes in tumorigenesis [6–10]. In addition to the numerous transfection experiments that have ascribed oncogenic [9,10] or anti-oncogenic [11] properties to specific PKC isoforms, some studies have reported tumour-associated genetic alterations of PKCα. Indeed a truncated PKCα (57 instead of 80 kDa) was found in a small carcinoma cell line from lung, probably resulting from aberrant post-translational processing [12]. Another altered form resulting from a tumour-specific deletion within the gene encoding PKCα was identified in a primary melanoma cell line [13]. More recently our laboratory identified a somatic point mutation leading to the replacement of an aspartic residue by glycine in position 294 of PKCα (mutation D294G) in human pituitary and thyroid tumours [14,15]. Overexpression of the D294G PKCα in Rat6 cells fails to induce growth in soft agar or tumour formation in nude mice but confers on Rat6 cells a relative independence from serum growth factors [16]. D294G PKCα is neither constitutively active nor inactive, because it is down-regulated as efficiently as the wild-type enzyme after prolonged treatment with PMA [16] and retains the capacity to phosphorylate histone IIIS or the epidermal growth factor receptor peptide [16]. However, the PMA-stimulated translocation of PKCα to the plasma membrane seems to be altered by the D294G mutation [16], which suggests an inability of the enzyme to reach and/or interact with its substrates, which are located in this cellular compartment.

With the use of recombinant wild-type and D294G PKCα produced by the baculovirus insect cell system, we show here that, although the mutant enzyme retains its catalytic properties, it exhibits a selective loss of substrate recognition on the basis of decreased binding of D294G PKCα to the recently cloned 35F and 35H PKC substrates [17,18]. These proteins, which have been identified as a novel C-terminal splice variant of kinesin light chain [17] and as a β-adducin homologue respectively, are known to exhibit properties of PKC-anchoring proteins [18]. Thus our findings suggest that, whereas D294G PKCα might retain its potential to translocate to the plasma membrane, its membrane association is probably not stabilized by PKCα–protein interactions.

EXPERIMENTAL

Preparation and isolation of recombinant PKC baculoviruses

The cDNA clones coding for both normal and D294G mutant human PKCα were from Dr I. B. Weinstein's laboratory (Columbia Cancer Center, New York, NY, U.S.A.) [16] and were subcloned into the pBacPAK9 vector purchased from Clontech.

The baculovirus *Autographa californica* nuclear polyhedrosis virus, containing the *LacZ* gene and *Bsu*36I restriction sites in the genes flanking the polyhedrin expression locus, was a gift from Dr. M. Martin and Dr. P. Mangeat (University of Montpellier II, Montpellier, France). DNA from pBacPAK9 transfer vectors (250 ng) containing normal or mutated PKCα genes and *Bsu*36I-digested baculovirus (7.5 μg) were co-transfected into *Spodoptera frugiperda* cells (10^6 Sf9 cells). Viral supernatants containing the recombinant baculoviruses were cloned with the plaque assay method in accordance with recommendations by Clontech.

Production and partial purification of normal and mutated PKCα from Sf9-infected cells

T-flasks containing 1.5×10^7 Sf9 cells were incubated at 27 °C for 3 days with 5×10^4 plaque-forming units/ml recombinant baculovirus. Viral supernatant was removed and monolayers of infected cells were recovered by using a stream of PBS [140 mM NaCl/27 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4 (pH 7.4)]. Normal and mutated PKCα were partly purified by the method of Birman et al. [19] with a DEAE-cellulose (DE-52) column. Elution conditions were established with a salt gradient ranging

Abbreviations used: PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C.

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from 50 to 200 mM NaCl. Each fraction was analysed for its PKCα content by Western blotting. In brief, 2 μl of each fraction was boiled for 10 min in the presence of SDS/PAGE sample buffer [25 mM Tris/HCl (pH 6.8)/10 % (w/v) SDS/10 % (v/v) glycerol/10 % (v/v) 2-mercaptoethanol/0.05 % Bromophenol Blue] and was loaded on a 10 % (w/v) polyacrylamide gel. Proteins were transferred to a PVDF membrane (NEN, Les Ulis, France) and were immunoblotted with a mouse monoclonal antibody against PKCα (dilution 1:2000) (UBI, Lake Placid, NY, U.S.A.) followed by a peroxidase-linked anti-(mouse IgG) antibody. Immunoblots were revealed by enhanced chemiluminescence reagent (Boehringer Mannheim, Indianapolis, IN, U.S.A.).

Enzymes were routinely eluted with 100 mM NaCl from the DE-52 column. Batch elutions containing either wild-type or D294G PKCα were stored at −80 °C in buffer D [final concentrations: 4 mM Tris, 200 μM EGTA, 200 μM dithiothreitol and 10 % (v/v) glycerol]. The content of PKCs from batch elutions was determined by the binding of phorbol 12,13-dibutyrate (PDBu) [20] and Western analysis to verify that equal amounts of PKC species calculated from PDBu-binding experiments were equated to identical signals from Western blots.

Isolectric pH measurement

Equal amounts (10 pmol) of wild-type PKCα and D294G PKCα were subjected to electrophoresis on a 5 % (w/v) polyacrylamide isoelectric focusing gel with a pH range from 3.5 to 10 [21]. Focusing was performed using 20 mM NaOH as catholyte buffer and 10 mM phosphoric acid as anolyte buffer [22]. Proteins were transferred to a PVDF membrane and were detected with the specific anti-PKC antibody, secondary antibody and ECL reagent mentioned above.

Binding of PDBu and catalytic activities of PKCα

Binding of PDBu was performed as described previously [20]. The same batch elutions of wild-type and D294G PKCα (obtained with 100 mM NaCl from the DE52 column and quantified by PDBu binding and Western blot analysis) were used for pI determinations, activity measurements and overlay experiments.

PKCα catalytic activity was measured by using histone IIIS (Sigma) and the S17R (SLKKRSGSFSKLRASIRR) and K18K (KKKKKFKTPSFLKKNKKK) peptides corresponding to the PKC phosphorylation sites of clones 35F and 35H respectively [17,18]. The cDNA for PKC-binding proteins 35F and 35H were isolated from a rat kidney expression library by using a PKC interaction cloning strategy. Homology searches indicate that 35F and 35H are a novel C-terminal splice variant of kinesin light chain [17] and a β-adducin homologue respectively [18]. Recombinant proteins were expressed from pQE (Qiagen) as a hexahistidine-tagged fusion protein and purified by nickel chromatography in accordance with the manufacturer's instructions (Qiagen) [17,18]. To determine the PKC phosphorylation sites in 35F and 35H, the fusion proteins were phosphorylated with PKC under standard conditions. The phosphorylated proteins were digested with trypsin and endoproteinase Lys-C, and peptides were separated by HPLC. Phosphorylated peptides were identified by liquid-scintillation counting and sequenced by automated Edman degradation. On the basis of these findings, the corresponding peptides S17R and K18K containing PKC phosphorylation sites were synthesized and used in PKC activity assays. Equal amounts (12 pmol/ml) of wild-type and D294G PKCα were mixed with various amounts of S17R or histone IIIS, 1 mM EGTA, 10 μM PMA, 5 mM magnesium acetate, 25 μM ATP, 1 mM dithiothreitol and 1 nM [γ-^{32}P]ATP (specific radioactivity 30 Ci/mmol) (Amersham, Little Chalfont, Bucks., U.K.). Phosphatidylserine (PtdSer) and Ca^{2+} EC_{50} values were determined by using PtdSer concentrations ranging from 0 to 30 μg/ml (in the presence of 1.2 mM Ca^{2+} and 10 μM PMA) and Ca^{2+} concentrations ranging from 0 to 1.2 mM (in the presence of 10 mg/ml PtdSer and 10 mM PMA) respectively. The reaction was started by incubating samples at 30 °C for 5 min; it was stopped at 0 °C for 5 min. Half the volume of each reaction sample was laid down on phosphocellulose paper P81 (Watman) squares; these were washed five times for 10 min in 0.01 M phosphoric acid, washed in acetone for 30 s and in light petroleum (boiling range 40–60 °C) for 10 s, then air-dried. Each square was then counted by liquid scintillation.

PKC overlay assay

Overlay assays were performed as described by Hyatt et al. [23]. Purified 35F and 35H proteins (150 ng) were applied directly to nitrocellulose with a slot-blot apparatus. Blots were incubated for 1 h with increasing concentrations of wild-type and D294G PKCα prepared in a buffer containing 20 mM Tris/HCl, 500 mM NaCl, 10 mg/ml BSA, 1 mM EGTA, 1.2 mM $CaCl_2$, 1 μg/ml PtdSer, 200 nM PDBu, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Semi-purified wild-type and D294G PKCα concentrations in the batch elutions obtained with 100 mM NaCl were determined by binding of PDBu (see above). It was also verified that the batch elutions led to identical signals in Western blot experiments (see Figure 4b). The effects of Ca^{2+} (0, 0.6 or 1.2 mM Ca^{2+} in the presence of 10 mg/ml PtdSer and 200 nM PDBu) or PtdSer (1 or 10 mg/ml in the presence of 1.2 mM Ca^{2+} with or without 200 nM PDBu) on the binding of wild-type and D294G PKCα to 35F and 35H were determined by using 0.3 pmol/ml PKCα proteins. Bound PKCα proteins were detected with a mouse monoclonal antibody against PKCα (dilution 1:2000) (UBI, Lake Placid, NY, U.S.A.) followed by a peroxidase-linked anti-(mouse IgG) antibody, revealed by enhanced chemiluminescence reagent (Boehringer Mannheim). Blots were quantified by scanning analysis with an NIH image program (version 1.59). Arbitrary units are defined as the product of the mean value of the pixels (between 0 and 255) and the number of pixels in the measured area. In all cases the background value was subtracted. All experiments were performed in duplicate.

RESULTS

To investigate the biochemical basis of the effects of the D294G mutation, wild-type and D294G PKCα were expressed in the baculovirus/insect cell system [24]. As expected, both wild-type and D294G PKCα were, as in mammalian cells, produced as 80 kDa forms after 72 h of infection (Figure 1a). However, with an increased duration of infection, a proteolytic PKCα form, also known as PKMα, was detected only in cells expressing D294G PKCα (Figure 1b). These differential proteolytic sensitivities of wild-type and mutant PKCα suggest that the D294G mutation, located in the V3 region and known to contain calpain as well as trypsin proteolytic sites [25], facilitates that cleavage.

Figure 2(A) shows that elution of the 80 kDa PKCα peak by anion-exchange chromatography occurred at 100 mM NaCl for both enzymes, whereas PKMα was eluted at 200 mM NaCl (Figure 2A, PKCα-mut). Therefore all further experiments were performed from a batch elution with 100 mM NaCl, which were therefore devoid of PKMα.

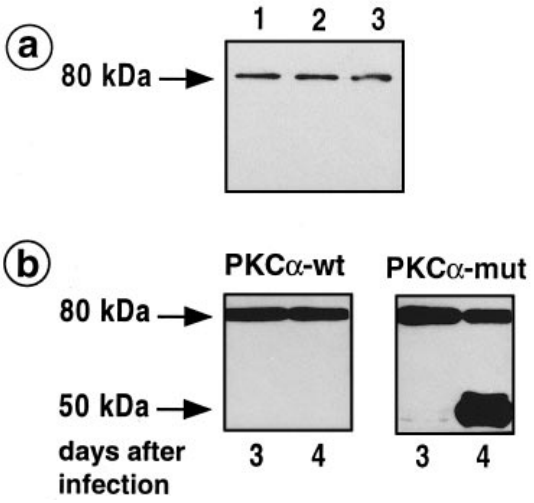

Figure 1 Recombinant wild-type and D294G PKCα are produced as fully phosphorylated enzymes

(**a**) Wild-type PKCα and D294G PKCα produced with the baculovirus insect cell system co-migrate with the 80 kDa PKCα overexpressed in mammalian cells. By using Western analysis [7% (w/v) polyacrylamide gel] the molecular masses of baculovirus-expressed wild-type PKCα (lane 1) and D294G PKCα (lane 2) were compared with that of wild-type PKCα overexpressed in stably transfected Rat 6 cells (lane 3). Note the absence of the 76 kDa unphosphorylated PKCα in all cases. (**b**) Wild-type (wt) PKCα and D294G (mut) PKCα expression with the baculovirus insect cell system. Sf9 cells were infected by baculoviruses containing cDNA species coding for either wild-type or D294G PKCα. After 3 and 4 days of infection (as indicated below the lanes), Sf9 cells were harvested and homogenized for an analysis of PKCα expression by Western blotting. Wild-type and D294G PKCα were detected with a monoclonal anti-PKCα antibody directed against the catalytic domain of PKCα and therefore recognizing native PKCα (80 kDa) as well as the 50 kDa catalytic domain resulting from hydrolysis within the V3 region. Note the presence of both PKCα forms in extracts containing D294G PKCα.

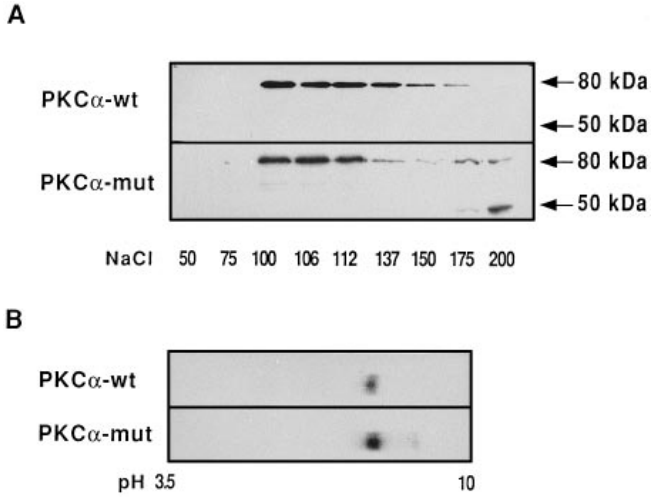

Figure 2 Comparative global charge of wild-type and D294G PKCα

(**A**) Wild-type (wt) and D294G (mut) PKCα were applied to an anion-exchange (DE-52) column and eluted with a 50–200 mM NaCl gradient. The resulting fractions were analysed for their PKCα content by Western blotting. Both native wild-type PKCα and D294G PKCα were eluted at comparable ionic strengths. Note that the 50 kDa PKCα proteolytic form, present only in D294G PKCα extracts, was eluted with an ionic strength distinct from that of the native enzyme. (**B**) Equal amounts of wild-type (wt) and D294G (mut) PKCα were subjected to an isoelectric focusing gel with a pH range from 3.5 to 10. Both PKCα proteins, which focused at a comparable pH (7.6), were then electrotransferred to PVDF membrane and detected with the monoclonal antibody against PKCα.

D294G PKCα retains its intrinsic catalytic potential

The affinities of wild-type and D294G PKCα for effectors (Ca^{2+}, PtdSer and PMA) were tested, with histone IIIS as substrate. As shown in Table 1, no significant differences in Ca^{2+}, PtdSer or PDBu activation constants were found between wild-type and D294G PKCα. Therefore the intrinsic catalytic potential is unaltered in the mutant protein. This finding also suggests that the PKCα mutant is transphosphorylated, as is the wild-type enzyme. Indeed, several pieces of evidence suggest that autophosphorylation events occur for the mutant enzyme: (1) immature fast-migrating (76 kDa) forms of the mutated enzyme were never detected in human tumours or in the previously stably transfected Rat6 cells overexpressing the mutant; (2) wild-type and D294G PKCα produced by the baculovirus co-migrate with the 80 kDa PKCα extracted from mammalian cells overexpressing PKCα in Western blot experiments with a 7% (w/v) polyacrylamide gel (Figure 1a); and (3) the comparable isoelectric points (7.6) of wild-type and D294G PKCα suggest that the global charge of the enzyme is not affected by the D294G mutation (Figure 2B). The latter results are in agreement with comparable elution profiles of the wild-type and mutant enzymes on anion-exchange chromatography (Figure 2B).

D294G mutation selectively affects PKCα interaction with substrates

Because wild-type and D294G PKCα exhibit similar lipid and Ca^{2+}-binding properties, the previously observed lack of plasma membrane translocation of D294G PKCα might be due to altered protein–protein interactions.

Table 1 PKCα effector dependence is unaltered by the D294G mutation

EC_{50} values for Ca^{2+} and PtdSer were determined with histone IIIS (2.5 mg/ml) as substrate: [Ca^{2+}] was varied from 0 to 1.2 mM in the presence of 10 μg/ml PtdSer and 10 μM PMA, and PtdSer concentrations varied from 0 to 30 mg/ml in the presence of 1.2 mM Ca^{2+} and 10 mM PMA. Binding of PDBu was measured by the method of Jaken and Kiley [20], with the use of a range of PDBu concentrations from 0 to 100 nM, in the presence of 1.2 mM Ca^{2+} and 10 μg/ml PtdSer. The two values given for each variable were obtained in two independent experiments.

PKCα type	Phosphatidylserine dependence, EC_{50} (μg/ml)		Ca^{2+} dependence, EC_{50} (mM)		PDBu binding, K_d (nM)	
Wild-type	1.10	1.30	0.89	0.95	7.75	7.92
D294G	1.05	1.20	0.90	0.97	13.34	11.11

Previous investigations on PKC interaction with plasma membranes, with the use of trypsin sensitivity, led to the discovery of a new class of PKC-interacting proteins involved in PKC compartmentalization [26]. Because these PKC-anchoring proteins include a series of PKC substrates [27], we compared the phosphorylating efficiencies of wild-type and D294G PKCα on the S17R (SLKKRSGSFSKLRASIRR) and K18K (KKKKK-FKTPSFLKKNKKK) substrates based on the recently cloned 35F and 35H proteins respectively [17,18] (S. Jaken, unpublished work). As shown in Figure 3, the S17R peptide is not phosphorylated by D294G PKCα even though it is a high-affinity substrate for wild-type PKCα. Comparable results were obtained with the K18K substrate (results not shown). The selective loss of substrate phosphorylation associated with D294G PKCα was not dependent on the concentration of activators. It was, however, dependent on the nature of the substrate, as attested by

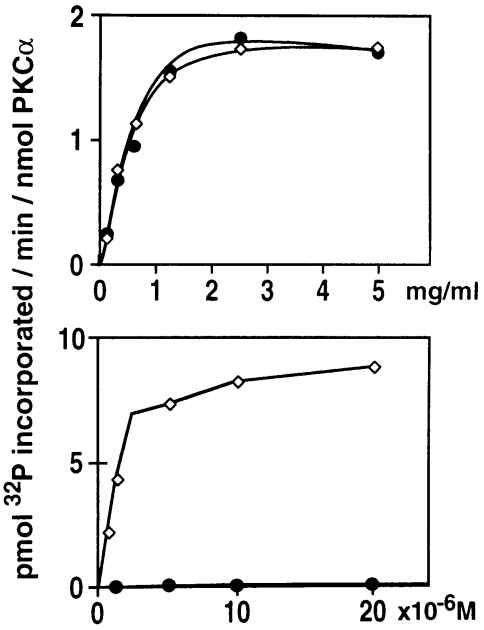

Figure 3 D294G PKCα exhibits a selective loss of substrate phosphorylation

PKC catalytic activities were measured with 0–5 mg/ml histone IIIS or 0–20 μM S17R (concentrations are shown along the abscissa) in the presence of 10 μg/ml PtdSer, 10 μM PMA and 1.2 mM $CaCl_2$. Whereas wild-type PKCα and D294G PKCα phosphorylated histone IIIS with comparable efficiencies (upper panel), the S17R substrate was not phosphorylated by D294G PKCα (lower panel) even though it is an excellent substrate for wild-type PKCα. Closely similar results were obtained in the three independent experiments performed. Symbols: ◇, wild-type PKCα; ●, D294G PKCα.

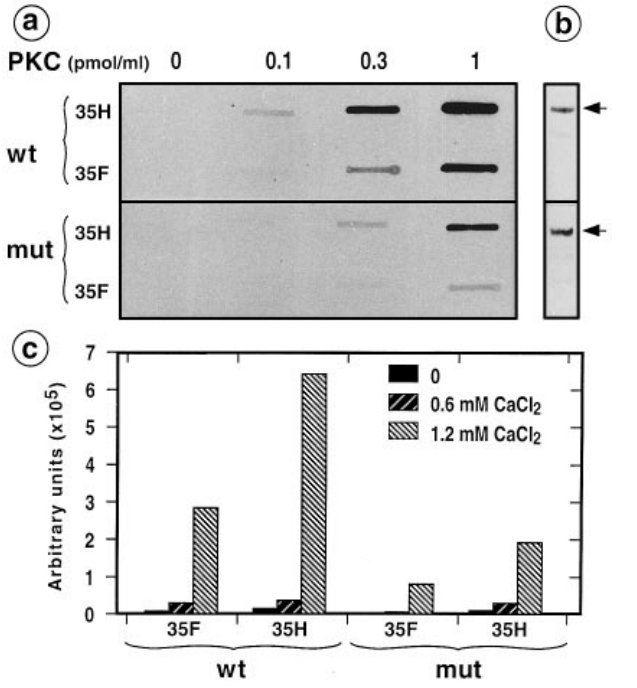

Figure 4 The D294G mutation induces a selective loss of PKCα substrate recognition

(**a**) Overlay experiments: the 35F substrate was blotted on a nitrocellulose membrane and incubated with various concentrations (0–1 pmol/ml) of wild-type (wt) PKCα or D294G (mut) PKCα in the presence of 10 μg/ml PtdSer, 200 nM PDBu and 1.2 mM $CaCl_2$. Bound PKCα proteins were detected with a monoclonal antibody against PKCα. D294G PKCα bound the 35F substrate with a lower affinity than did the wild-type enzyme. (**b**) Western blot of wild-type PKCα (upper panel) and D294G PKCα (lower panel) semi-purified extracts used for overlay experiments. Note that both extracts contained equal amounts of enzyme, as expected from PDBu binding. (**c**) Effects of Ca^{2+} on the binding of wild-type (wt) and D294G (mut) PKCα to 35F and 35H were determined as described in (**a**) with 0.3 pmol/ml PKCα proteins in the presence of 10 μg/ml PtdSer and 200 nM PDBu. Blots were quantified by scanning analysis. Neither increasing concentrations of PtdSer and Ca^{2+} nor the presence of PDBu enhanced the binding efficiency of D294G PKCα. Closely similar results were obtained in the two independent experiments performed.

comparable D294G and wild-type PKCα efficiencies in phosphorylating histone IIIS (Figure 3) or the epidermal growth factor receptor peptide [16]. The possibility that D294G PKCα might have an altered substrate-binding affinity had then to be considered. Therefore both recombinant enzymes were employed to test substrate binding, with a blot overlay approach. As shown in Figure 4(a), D294G PKCα bound recombinant 35F and 35H proteins with a much lower affinity than did the wild-type enzyme. The slightly different binding efficiency of PKCα for the 35F and 35H substrates was not correlated with different phosphorylation efficiencies (results not shown). In agreement with phosphorylation experiments, substrate binding by D294G PKCα was not restored by increasing Ca^{2+} (Figure 4c), PtdSer or PDBu (Table 2) concentrations, establishing that the D294G point mutation affects PKCα substrate specificity rather than catalytic properties themselves. Furthermore the apparent increased interaction between D294G PKCα and 35H substrate in the presence of 10 μg/ml PtdSer was not correlated with an increased phosphorylation efficiency of the corresponding K18K peptide compared with the S17R peptide: labelled phosphate (^{32}P) incorporation into K18K (20 μM) measured with equal amounts of wild-type PKCα and D294G PKCα was 30000 c.p.m. for wild-type PKC and 250 c.p.m. for the mutant; phosphorylation of S17R (20 μM) was 43000 c.p.m. with wild-type enzyme and 300 c.p.m. with the mutant.

DISCUSSION

This study establishes that the tumour-associated D294G PKCα mutant retains its catalytic potential on the basis of comparative binding affinities for Ca^{2+}, phosphatidylserine and PDBu between wild-type and D294G PKCα, with histone IIIS as substrate. This finding is consistent with the fact that the mutation is not located in the regulatory domain, which is known to contain interaction sites with PKC effectors. This result, together with the identical apparent molecular masses (80 kDa) and isoelectric points (7.6) of normal and mutant PKCα, also suggests that the D294G PKCα mutant is both transphosphorylated and autophosphorylated [28,29]. This indicates that the D294G PKCα mutant retains its autocatalytic potential. Indeed, PKCα is synthesized as an unphosphorylated and catalytically inactive protein of 76 kDa that is first phosphorylated on Thr-497 and Ser-657 by hitherto unknown heterologous kinases [28]. This first stage of phosphorylation is a preliminary event required for the activation of PKC. A second stage of phosphorylation, occurring on Thr-638, takes place as an autophosphorylation event that controls the accumulation of an 80 kDa active enzyme and also contributes to its resistance to phosphatases [29]. However, the mutant enzyme exhibits a selective loss of catalytic activity towards the synthetic peptides S17R and K18K, which are based on the recently identified PKC substrates 35F and 35H. Overlay experiments clearly demonstrate that the lack of phosphorylation of S17R and K18K peptides is correlated with a selective loss of

Table 2 Effect of PDBu and PS on binding of wild-type PKCα and D294G PKCα to 35F and 35H

Blots were prepared as described in Figure 4(a) and incubated in the presence of 0.3 pmol/ml PKCα proteins with 1.2 mM Ca^{2+}. The presence of PDBu (200 nM) or PtdSer (1 or 10 μg/ml) did not influence the binding efficiency of D294G PKCα. Results are arbitrary units obtained by scanning analysis (NIH image program, version 1.59) and represent the product of the mean value of the pixel (between 0 and 255) and the number of pixels in the measured area. In each case the background value was subtracted. Results were obtained from a representative experiment of the two performed.

			Binding (10^{-5} × arbitrary units)			
			Wild-type PKCα		D294G PKCα	
[PtdSer] (μg/ml)	PDBu	Binding to …	35F	35H	35F	35H
1	−		15.2	30.5	9.8	19.1
	+		98.4	87.6	29.1	48.4
10	−		39.7	74.0	19.6	32.2
	+		90.6	112.8	30.0	69.1

affinity of D294G PKCα for the corresponding 35F and 35H proteins. The selective loss of substrate recognition exhibited by D294G PKCα suggests that the V3 hinge region has a critical role in substrate recognition. However, recent studies indicate that important determinants for isotype-specific function are, as expected, located within the catalytic domain. Indeed, the chimaera containing the regulatory domain of PKCα and the catalytic domain of PKCβ1 confers the biological function of PKCβ1 on human erythroleukaemia cells, whereas the reverse chimaera containing the regulatory domain of PKCβ1 and the catalytic domain of PKCα behaves like PKCα [30]. Because it has no significant effects on the enzyme's affinity for activators, the D294G point mutation might selectively affect substrate interactions through a conformational change in the catalytic domain. However, a global modification in the conformation of the enzyme is more likely to occur. Indeed, the V3 region is suspected to be important for the flexibility of the enzyme, suggesting that subtle conformational modifications within this domain, evidenced here by the increased proteolytic sensitivity of the mutant enzyme (see Figure 1b), might have significant effects on the overall conformation of the protein rather than on the conformation of individual domains. This conclusion is consistent with previous binding studies performed with the 35F substrate, showing that at least two different sites are implicated in the binding of PKCα to this protein: the first is located within the pseudosubstrate, which, if deleted, leads to a decreased binding to 35F [17]; the second might be located in the catalytic core, as these proteins are also PKC substrates. Thus the D294G mutation might alter a specific conformation of PKCα in which the pseudosubstrate is located in the vicinity of the catalytic domain and results in an increased affinity of the enzyme for such substrates.

The consequence of this loss of protein–protein interactions is likely to be the aberrant subcellular location of the activated D294G PKCα reported in our previous paper [16]. Indeed, the present results suggest that the mutant enzyme might retain its potential to translocate to the plasma membrane under stimulated conditions but that this membrane association might not be stabilized through interactions between PKC and anchoring protein. The 35H and 35F proteins represent candidate proteins for stabilizing the membrane localization of PKCα because their binding to PKCα is effector-dependent and involves a domain distinct from the catalytic core. Moreover, PKCα has been found to be co-localized with 35H in the cell–cell junction of renal proximal tubule epithelial cells [27]. It remains to be determined whether this specific loss of PKCα–protein interaction is restricted to these proteins, or whether the lack of membrane translocation of D294G PKCα is due to a more general loss of PKCα–protein interactions involving other predicted anchoring proteins such as annexins, myristoylated alanine C-kinase substrate (MARCKS) or receptors for activated proteins (RACKs) [31].

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