Comparison of the roles of the C1a and C1b domains of protein kinase C alpha in ligand induced translocation in NIH 3T3 cells

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Received 25 May 1999

Abstract To explore the relative roles of the two C1 domains of protein kinase C alpha (PKCα) in the response to phorbol esters and related analogs, we mutated the individual C1 domains, expressed the mutated PKCα in NIH 3T3 cells, and then examined the ability of ligands to induce its translocation to the membrane. The C1a and C1b domains play equivalent roles for translocation in response to phorbol 12-myristate 13-acetate, mezerein, and (−)octylindolactam V. These results contrast with those previously reported for PKCδ, suggesting that the domains play different roles in different PKC isoforms.

Key words: Phorbol 12-myristate 13-acetate; PMA; Tumor promotion; sn-1,2-Diacylglycerol

1. Introduction

The lipophilic second messenger sn-1,2-diacylglycerol signals through interaction with the C1 domains present on protein kinase C (PKC) isoforms [1,2], as well as on the chimaerins [3,4] and on RasGRP [5]. Several structurally distinct classes of natural products including the phorbol ester tumor promoters and the indole alkaloids function as potent analogs of diacylglycerol, likewise interacting with the C1 domains [6]. The structure of the complex between the second C1 domain of PKCδ and phorbol 13-acetate has been solved by X-ray crystallography [7], and insight into the interactions with other ligands has been provided by computer modeling [8].

Unlike the chimaerins and RasGRP, PKC possesses tandem C1 domains. The relative involvement of these two C1 domains in the response to the phorbol esters and related ligands remains uncertain, however. Using isolated C1 domains, Irie et al. [9] have suggested that only the C1b domain of most PKC isoforms, including PKCα, recognizes phorbol esters. In contrast, studies using intact PKC and a variety of methodological approaches have suggested that both domains function, but with varying contributions depending on the specific ligand [10–13].

The approach we have taken has been to mutate either the C1a, C1b, or both domains so as to reduce the affinity of interaction without abolishing activity or causing gross disruption of the structure. The mutated PKC has then been expressed in NIH 3T3 cells and its response to ligand monitored by the ability of ligand to induce translocation from the cytosol to the membrane. Using PKCδ, we have reported that translocation in response to phorbol 12-myristate 13-acetate (PMA) depends on the C1b domain, but with a further contribution by the C1a domain evident in the double mutant [14]. The indole alkaloid (−)octylindolactam V (octyl-ILV) showed behavior similar to that for PMA [13]. Mezerein, in contrast, showed equivalence between the two C1 domains, with either the C1a or C1b mutants showing a comparable decrease in potency for translocation [13]. Shieh et al., using deletions of either the C1a or C1b domains of PKCα, had likewise described different dependence on the C1a and C1b domains for different ligands, using effects on cell proliferation in yeast as an endpoint [11,12]. However, their conclusions differed regarding the pattern of dependence for different ligands. Thus, in their studies PMA showed equal dependence on C1a and C1b, mezerein depended on C1a, and octyl-ILV required both C1a and C1b for activity. These differences could have reflected the isoform, the cell system, or the nature of the alterations in the C1 domains. In the present study, we have extended our previous approach to PKCα. We conclude that different PKC isotypes, under parallel conditions, show different dependence on the C1a and C1b domains for ligand driven translocation.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA), mezerein, and (−)octylindolactam V were purchased from Alexis Corporation (San Diego, CA, USA).

2.2. Site-directed mutagenesis of PKCα

The bovine PKCα was subcloned by polymerase chain reaction (PCR) into the pGEM-T vector (Promega, Madison, WI, USA) using the 5′ oligonucleotide CGCTCGAGATGGCTGACGTCTTCC (containing the underlined *Xho*I site) and the 3′ oligonucleotide CGACGCGTTACCGCGCTCTGCAGGATGG (containing the underlined *Mlu*I site). The PCR reaction was carried out for eight cycles using the high fidelity thermostable Vent DNA Polymerase (New England Biolabs, Beverly, MA, USA). The fidelity of the PCR reaction was confirmed by direct sequencing. This plasmid served as our 'master' vector for the subsequent mutagenesis. Site-directed mutagenesis was performed using the Transformer Site-directed Mutagenesis Kit from Clontech (Palo Alto, CA, USA). The following oligonucleotides served as primers for selection and mutagenesis: (1) 5′-CGCCTGCAGATCGATCATATGGGAGAG-3′ changes the *Sal*I site present in the pGEM-T vector at position 75 into the underlined *Cla*I restriction site. (2) 5′-CGCTTCTTCAAGCAG**GGT**ACCTTCTGCAGC-3′ mutates the proline residue at position 47 into glycine (bold letters) and creates the underlined *Kpn*I restriction site to facilitate the detection of mutant plasmids. (3) 5′-ACGTATGGCAGC**GGT**ACCTTCTGTGAT-3′ mutates the proline residue in position 112 into glycine (bold letters) and creates the underlined *Kpn*I restriction site to facilitate the detection of mutant plasmids. The mutations were confirmed by direct sequencing (Paragon Biotech Inc., Baltimore, MD, USA). The single mutants proline to glycine at site 47 (P47G), proline to glycine at site 112 (P112G), and the double mutant (P47/112G) were subcloned along with the wild-type into an epitope tagging mammalian expression vector described in

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PII: S0014-5793(99)00927-8

detail by Olah et al. [15]. The *Xho*I and *Mlu*I restriction sites ensure unidirectionality and the vector attaches to the end of PKCα a C-terminal 12 amino acid tag, originally derived from the C-terminal sequence of PKCε.

2.3. Cell culture and transfection of cells

NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 4500 mg/l glucose, 4 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Advanced Biotechnologies Inc., Columbia, MD, USA) and 10% fetal calf serum (complete DMEM) (Gibco BRL-Life Technologies, Gaithersburg, MD, USA). The cells were transfected with the wild-type and mutant constructs using Lipofectamine (Gibco BRL-Life Technologies, Gaithersburg, MD, USA) and selected as described previously [16]. For the assessment of the amounts of soluble and membrane-associated mutant and wild-type PKCα, the cells were incubated with different concentrations (1 nM–30 μM) of PMA, mezerein, and octyl-ILV (Alexis Corp. San Diego, CA, USA) for 30 min, 1 h, 3 h, or 6 h at 37°C. All compounds were dissolved in dimethyl sulfoxide (DMSO) such that the final concentration of DMSO in the medium was 0.1%, including for the control cells.

2.4. Cell lysis and Western blot analysis

The cells were harvested and lysed, and the soluble and Triton X-100-soluble particulate fractions were prepared as described previously [16]. The protein content of the cytosolic fraction was measured by a micromethod using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Twelve μg protein of the cytosolic fraction were mixed with equal volumes of 2×SDS sample buffer (125 mM Tris-Cl, pH 6.8, 20% glycerol, 4% SDS, 0.71 M β-mercaptoethanol and bromophenol blue) and subjected to SDS-PAGE using 8% polyacrylamide gels (Novex, San Diego, CA, USA) followed by electrotransfer onto nitrocellulose membranes. The membranes were immunostained using the polyclonal anti-PKCε antibody (Gibco BRL-Life Technologies, Gaithersburg, MD, USA) applied as described previously [16]. Densitometric analysis was performed under conditions that yielded a linear response and analyzed using the National Institutes of Health Image 1.52 program (written by Dr. Wayne Raspband, National Institutes of Health).

3. Results and discussion

We compared the ability of ligands to induce translocation of PKCα wild-type and mutants using epitope-tagged enzyme expressed in NIH 3T3 cells. We have previously described that neither the overexpression nor the epitope tag affect the ED_{50} of PKCα for translocation; likewise, they do not affect the subcellular fraction to which it translocates [13,14,16]. Translocation was quantitated by Western blotting, assessing the decrease in the level of PKCα present in the soluble fraction. The level of total PKCα was monitored in parallel to confirm that observed decreases were not due to down regulation of the enzyme over the time period examined. All data reflect 4–6 independent experiments.

Measurement of translocation was determined as a function of dose of ligand and of time. The three ligands examined were PMA, mezerein, and octyl-ILV. The incubation times were 30 min, 1, 3, and 6 h. For all three ligands, the dose response curves were independent of time by 3 h, and at this time the level of PKCα had not yet begun to decrease due to down regulation over the range of ligand concentrations defining the curves (data not shown). The dose response curves for translocation, representing the averaged data for the 3 h incubation, are illustrated in Fig. 1. Representative Western

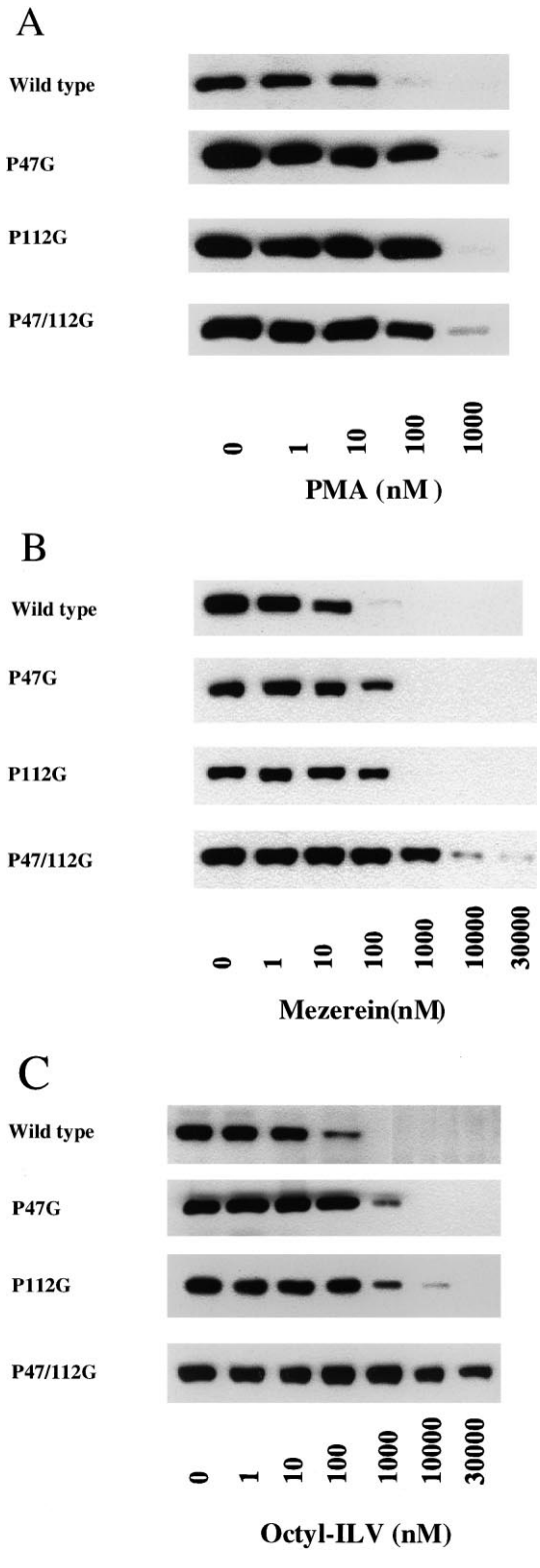

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Fig. 1. PMA (A), mezerein (B), and octyl-ILV (C) induced changes in levels of wild-type and mutant PKCα in the cytosolic fraction of NIH 3T3 fibroblasts. NIH 3T3 fibroblasts transfected with the wild-type or mutant forms of PKCα were treated with the indicated doses of PMA, mezerein, or octyl-ILV for 3 h. The soluble fraction was prepared for SDS-PAGE, and Western immunoblotting was performed as described in Section 2. The amount of the enzyme was quantitated by densitometry and expressed as the percentage of the amount of isozyme present in the soluble fraction in the control. The illustrated *curves* are calculated from the Hill equation. *Points* represent the average of four to six independent experiments ± S.E.M.

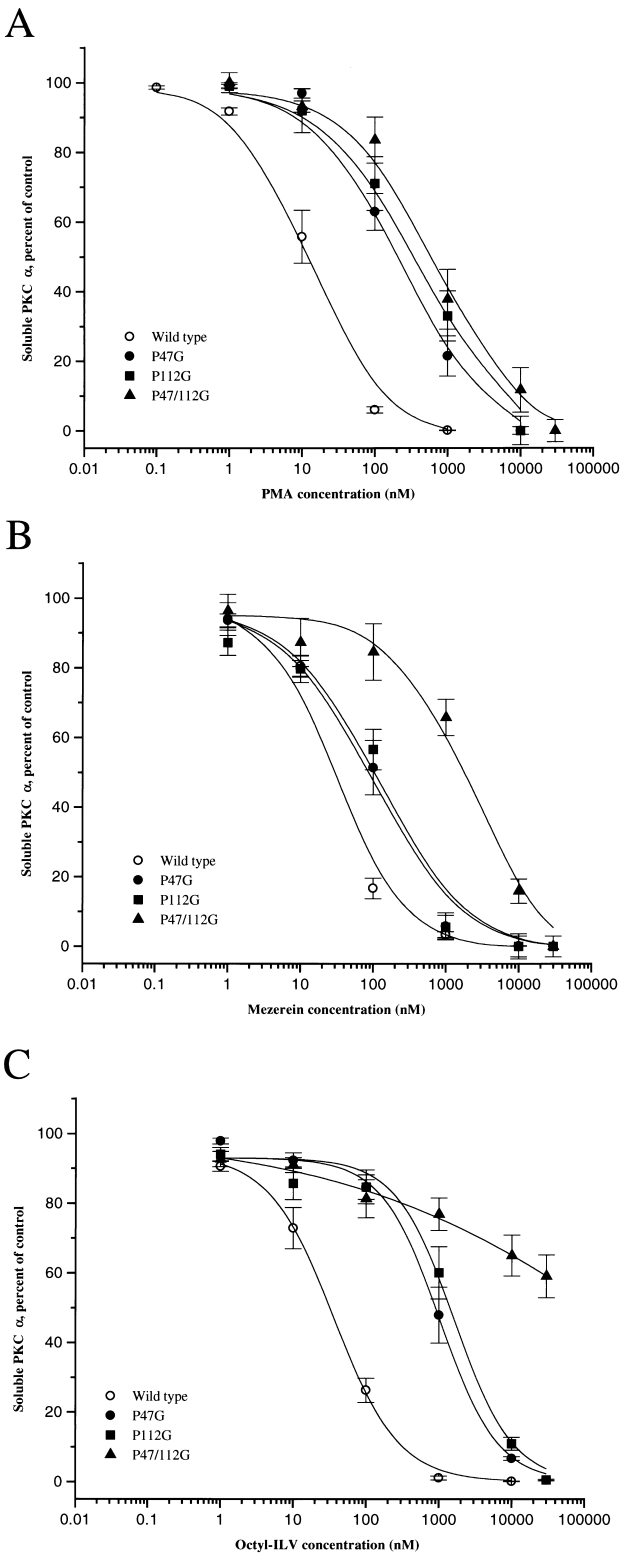

Fig. 2. Dose-dependent translocation of the wild-type and mutant forms of PKCα by PMA (A), mezerein (B), and octyl-ILV (C). NIH 3T3 fibroblasts overexpressing the wild-type or mutant forms of PKCα were treated by the indicated doses of PMA, octyl-ILV, and mezerein for 3 h. The soluble fraction was prepared for SDS-PAGE, and Western immunoblotting was performed as described in Section 2. Equal amounts of protein of the cytosolic fraction (12 μg of protein/lane) were loaded in each lane. Similar results were obtained in four–six sets of independent experiments for each ligand.

blots are shown in Fig. 2. The ED_{50} values are summarized in Table 1.

PMA translocated wild-type PKCα with an ED_{50} of 12 nM (Fig. 1A). Mutations in either the C1a or the C1b domain caused shifts of 18-fold and 31-fold, respectively. It is thus clear that both C1 domains make a major contribution to PMA induced translocation, although there is a modestly greater contribution by the C1b domain. For the PKCα double mutant, there was only a small (2–3-fold) additional shift in the ED_{50} for PMA compared to that of the single mutants. These results contrast markedly with those obtained previously for PKCδ [14]. In that case, the shifts in ED_{50} had been 1.1- and 18-fold for the C1a and C1b mutants, respectively, and the C1a/C1b double mutant had shown a further shift in its ED_{50} to 127-fold that of the wild-type.

Mezerein translocated wild-type PKCα with an ED_{50} of 33 nM; it was thus slightly less potent than PMA (Fig. 1B). Mutations in the C1a and C1b domains caused closely comparable 3- and 4-fold shifts. The double mutant showed a 60-fold shift in ED_{50}. The results of the single mutants are qualitatively similar to those observed with PKCδ [13], where the two C1 domains were basically equivalent, with 14- and 18-fold shifts. Although the double mutant behaved in a qualitatively similar fashion for PKCα and PKCδ, quantitatively the loss of affinity was less for PKCα. In the case of PKCδ, negligible decrease was detected up to 30 μM.

Octyl-ILV translocated wild-type PKCα with an ED_{50} of 36 nM (Table 1). It thus is similar in potency to mezerein. The mutations in the C1a and C1b mutations caused comparable 29- and 45-fold shifts in ED_{50}, respectively. The double mutant displayed an additional shift, but its magnitude could not be assessed because of the weak response. In the case of PKCδ, the C1 domains had not been equivalent for recognition of octyl-ILV [13]. The mutants in the C1a and C1b domains had displayed shifts of 1.5- and 20-fold, respectively, analogous to the behavior of PMA [13,14]. The shift for the double mutant had been 364-fold. Octyl-ILV thus resembles PMA in the difference in its C1 domain dependence between PKCα and PKCδ, shifting from dependence primarily on C1b in PKCδ to equivalence of C1a and C1b in PKCα.

Our findings have several important implications. First, it is clear that all PKC isoforms cannot be treated as equivalent with regard to the role of the C1 domains. Rather, for detailed understanding of their function and involvement in structure-activity relations, the behavior of the individual isoforms will need to be individually characterized. This perhaps should not be a surprise, in that the isoforms differ with regard to many of their other structural properties. PKCδ

Table 1
Potencies of PMA, mezerein, and octyl-ILV to induce translocation of the wild-type and mutant forms of PKCα

	ED_{50} (nM)		
	PMA	Mezerein	Octyl-ILV
PKCα wild-type	12 ± 1	33 ± 6	36 ± 4
PKCα P47G	216 ± 29	100 ± 20	1030 ± 110
PKCα P112G	368 ± 59	119 ± 23	1620 ± 180
PKCα P47/112G	644 ± 88	1970 ± 370	n.d.

ED_{50} values were determined from fitting of the data to the Hill equation. The ED_{50} ± S.E.M. values were derived from four to six independent experiments.
n.d., not determined.

shows different dependence on phosphorylation in the activation loop for activation of kinase activity [17]. PKCε has been reported to show distinct phospholipid dependence [18]. PKCη is selectively activated by cholesterol sulfate [19].

We had described previously that the ability of ligands to cause translocation of PKC depends not only on the C1 domain but also on the molecular context in which that domain is found [16]. PKCα was induced to translocate with 30-fold weaker ED_{50} than was a chimera containing the regulatory domain of PKCα and the catalytic domain of PKCε [16]. Presumably, the mechanism is related to the coupling between the conformational changes associated with ligand binding and translocation and the contributions of other structural elements in PKC to the stabilization of the different conformations. Oancea and Meyer have elegantly demonstrated contributions to translocation of PKCγ by the pseudosubstrate region and the C2 domain as well as by the C1 domains [20]. Such differences may contribute to the different behavior of different isoforms.

The C1 domains of the various diacylglycerol/phorbol ester receptors show substantial conservation. The roles of other structural features in determining the response of the intact proteins to their ligands provides abundant further opportunities for the establishment of selectivity. An on-going challenge is to understand these differences at the mechanistic level, so that they can be exploited in a rational rather than simply an empirical fashion.

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