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Non-equivalent Roles for the First and Second Zinc Fingers of Protein Kinase Cδ

EFFECT OF THEIR MUTATION ON PHORBOL ESTER-INDUCED TRANSLOCATION IN NIH 3T3 CELLS*

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Classical and novel protein kinase C (PKC) isozymes contain two, so-called cysteine-rich zinc finger domains that represent the binding sites for phorbol esters and the diacylglycerols. X-ray crystallographic, mutational, and modeling studies are providing detailed understanding of the interactions between the phorbol esters and individual PKC zinc fingers. In the present study, we explore the roles of the individual zinc fingers in the context of the intact enzyme. Our approach was to mutate either the first, the second, or both zinc fingers of PKCδ, to express the mutated enzyme in NIH 3T3 cells, and to monitor the effect of the mutations on the dose-response curve for translocation induced by phorbol 12-myristate 13-acetate. The introduced mutations change into glycine the consensus proline in the phorbol ester binding loop of the zinc finger; in the isolated zinc finger, this mutation causes a 125-fold decrease in phorbol ester binding affinity. We observed that mutation in the first zinc finger caused almost no shift in the dose-response curve for translocation; mutation in the second zinc finger caused a 21-fold shift, whereas mutation in both zinc fingers caused a 138-fold shift. We conclude that the zinc fingers in the intact PKC are not equivalent and that the second zinc finger plays the predominant role in translocation of protein kinase Cδ in response to phorbol 12-myristate 13-acetate. Our findings have important implications for the understanding and design of PKC inhibitors targeted to the zinc finger domains.

Protein kinase C (PKC)[1] comprises a family of isozymes that mediate signal transduction for the lipophilic second messenger diacylglycerol, regulating a wide array of cellular processes (see Refs. 1 and 2 for review). PKC possesses two functional domains, an N-terminal regulatory domain and a C-terminal catalytic domain. Within the regulatory domain lie two cysteine-rich zinc fingers, responsible for recognition by PKC of diacylglycerol or their ultrapotent analogs, the phorbol esters. In isolation, the individual first and second zinc fingers bind phorbol ester with similar affinities (3, 4), and the molecular details of the interaction between the phorbol esters and the zinc finger domain are beginning to emerge. NMR spectroscopy has yielded the solution structure of the second zinc finger of PKCα (5, 6); the structure of the complex between the second zinc finger of PKCδ and phorbol 13-acetate has been solved by x-ray crystallography (7). Site-directed mutagenesis has further highlighted key residues within the zinc finger structure required for ligand binding (8), and computer modeling is providing insight into how other high affinity ligands, *e.g.* the indole alkaloids, interact with the zinc finger.

An issue that is only beginning to be addressed is the role of the individual zinc fingers within the context of the intact PKC molecule. In pioneering studies, Bell and co-workers (9) had reported that the binding affinity of the second zinc finger of PKCγ was reduced in constructs containing sequences C-terminal to the zinc finger. Riedel and co-workers (10) analyzed the effect of the deletion of the first or second zinc finger of PKCα in a phenotypic yeast assay; they reported that PMA showed a comparable loss in potency for activation of the deletion mutants at either site; mezerein activated predominantly through the first zinc finger, whereas (−)-indolactam V required both intact zinc fingers for activity. Unfortunately, interpretation of the results of deletion analysis is clouded by the extent of potential disruption of the structure of the holoenzyme; for example, the deletion of the first zinc finger also included the pseudosubstrate domain immediately N-terminal to it.

As an alternative approach, we have introduced a proline to glycine mutation into the first or second or both zinc fingers of PKCδ. We had previously reported that this mutation, in the isolated zinc finger, preserves binding activity but causes a substantial loss (125-fold) in binding affinity (8). We evaluated the quantitative influence of these mutations on recognition of phorbol 12-myristate 13-acetate in the intact cell, using as an end point translocation of the mutant PKC from the soluble to the detergent-soluble particulate fraction. We report here that the zinc fingers in the intact PKC are not equivalent for inducing translocation and that the second zinc finger in the intact PKC has approximately 25-fold higher affinity for PMA than does the first zinc finger under these cellular conditions.

EXPERIMENTAL PROCEDURES

Phorbol 12-myristate 13-acetate was purchased from LC Laboratories (Woburn, MA) and dissolved in ethanol.

For site-directed mutagenesis of PKCδ, mouse PKCδ (a kind gift of H. Mischak and F. J. Mushinski) was cloned into the pGEM-T vector (Promega, Madison, WI) as described previously (11). This plasmid served as our "master" vector for site-directed mutagenesis using the same selection primer as described in Ref. 11. The following oligonucleotides served as mutagenesis primers. 1) 5′-ACCTTCTTC-GGGCAG**GGT**ACCTTCTGTTCT-3′ mutates the proline residue at position 169 into a glycine (bold letters) and creates the underlined *Kpn*I restriction site to facilitate the detection of mutant plasmids. 2) 5′-AACTACATGAGC**GGT**ACCTTCTGTGACCAC-3′ mutates the proline residue at position 241 into a 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). The single mutants proline to glycine at site 169 (P169G), proline to glycine at site 241 (P241G), and the double mutant (P169G/P241G) were subcloned, along with the wild type, into an epitope tagging mammalian expression vector described elsewhere

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[1] The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.

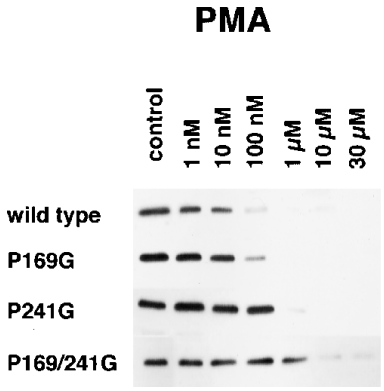

FIG. 1. **Dose-dependent translocation of the wild type and mutant forms of PKCδ by PMA.** NIH 3T3 fibroblasts transfected with the wild type or mutant forms of PKCδ were treated by the indicated doses of PMA for 60 min. The soluble fraction was prepared for SDS-PAGE, and Western immunoblotting was performed as described under "Experimental Procedures." Equal amounts of protein of the cytosolic fraction (10 μg of protein/*lane*) were loaded in each *lane*. Identical results were obtained in three independent sets of experiments.

(12). The constructs were transfected into NIH 3T3 fibroblasts using Lipofectamine (Life Technologies, Inc.) and selected as described elsewhere (11).

For the assessment of the amounts of soluble and membrane-associated mutant and wild type PKCδ, the cells were routinely treated with the indicated doses of PMA for 10 min, 30 min, 1 h, and 6 h at 37 °C. The cells were harvested and lysed, and the soluble and Triton X-100-soluble particulate fractions were prepared as described previously. The protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The protein content of individual samples was determined (13), and the blots were stained using the polyclonal anti-PKCε antibody from Life Technologies, Inc. as described previously (13). The densitometric analysis of the immunoblots and the normalization to the protein content of each individual lane were performed as described (13).

RESULTS AND DISCUSSION

Using a previously described tagging system (12) we could readily distinguish the endogenous and overexpressed PKCδ. The tagged, overexpressed wild type PKCδ translocated with a time course and dose-response curve similar to that of the endogenous enzyme (data not shown), arguing against artifacts caused by the overexpression (13). Previously we had shown that the kinetics of translocation in response to various PKC activators depended on the PKC isozyme and the cellular system (14). Under the present conditions, the translocation dose-response curves for the mutant and wild type PKC δ were stable by 60 min of exposure to PMA, and at this time point down-regulation was not yet detectable (data not shown and Ref. 13). We therefore determined the dose-response curves for translocation at this time point (Fig. 1). Data were fitted to the Hill equation.

PMA translocated the wild type form of PKCδ with an ED_{50} of 13 ± 1 nM, showing excellent correlation with the data obtained for the endogenous enzyme in a previous publication (13). Mutation in the first zinc finger (P169G) caused a barely detectable 1.1-fold shift. Mutation in the second zinc finger alone (P241G) caused an approximately 21-fold shift, whereas mutations in both zinc fingers caused an approximately 138-fold shift (Fig. 2, Table I). Qualitatively, these data demonstrate that the two zinc fingers are not equivalent and the second zinc finger is the primary contributor to translocation of the native enzyme.

Quantitatively, the observed behavior is consistent with the following model. We assumed that PKC exists in four possible states in the presence of phorbol esters: 1) when no phorbol ester is bound to any of the zinc fingers (P_0); 2) when only the

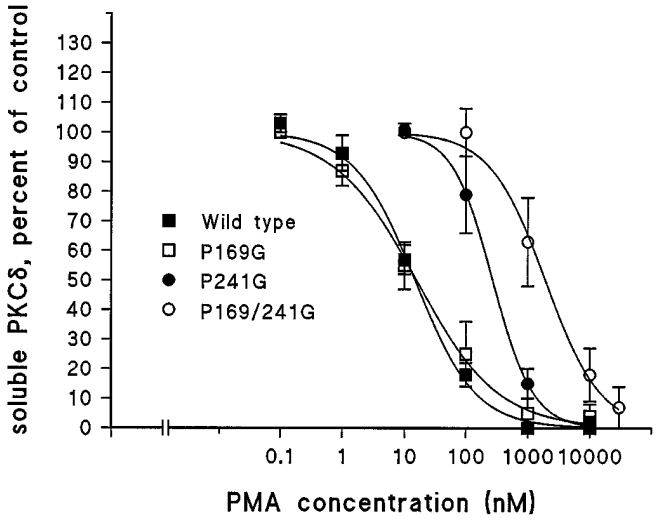

FIG. 2. **PMA-induced changes in levels of wild type and mutant PKCδ in the cytosolic fraction of NIH 3T3 fibroblasts.** NIH 3T3 fibroblasts transfected with the wild type or mutant forms of PKCδ were treated by the indicated doses of PMA for 60 min. The soluble fraction was prepared for SDS-PAGE, and Western immunoblotting was performed as described under "Experimental Procedures." 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 independent experiments ± S.E.

TABLE I
Potency of PMA to induce translocation of the wild type and mutant forms of PKCδ

ED_{50} and Hill coefficient values were determined from fitting of the data to the Hill equation or the kinetic model (Equation 5). The ED_{50} and Hill coefficient values ± S.E. were derived from four independent experiments.

	Fit to Hill equation		Fit to kinetic model	
	ED_{50}	Hill coefficient	ED_{50}	Hill coefficient
	nM		nM	
PKCδ wild type	15 ± 0.6	0.9 ± 0.03	13.6	1.10
PKCδ P169G	16 ± 0.5	0.7 ± 0.02	15	1.00
PKCδ P241G	270 ± 10	1.35 ± 0.04	250	1.22
PKCδ P169G/P241G	1900 ± 70	1.0 ± 0.03	1660	1.10

first zinc finger binds phorbol ester (P_1); 3) when only the second zinc finger binds phorbol ester (P_2); and 4) when both zinc fingers bind phorbol ester ($P_{1,2}$). Under equilibrium conditions the following equations describe the amount of PKC in the individual states,

$$[L]\ [P_0] = k_1\ [P_1] \quad \text{(Eq. 1)}$$

$$[L]\ [P_0] = k_2\ [P_2] \quad \text{(Eq. 2)}$$

$$[L]\ [P_1] = k_3\ [P_{1,2}] \quad \text{(Eq. 3)}$$

$$[L]\ [P_2] = k_4\ [P_{1,2}] \quad \text{(Eq. 4)}$$

where [L] is the concentration of the free phorbol ester, and k_1 through k_4 are the apparent dissociation constants for the individual equilibria.

Assuming that binding of phorbol ester to any or both zinc fingers causes translocation, the ratio of translocated to total PKC is described by the following equation.

$$\frac{[P_1] + [P_2] + [P_{1,2}]}{[P_0] + [P_1] + [P_2] + [P_{1,2}]} = \frac{\frac{[k_3]}{[L]} + \frac{[k_4]}{[L]} + 1}{\frac{[k_3]}{[L]} + \frac{[k_4]}{[L]} + \frac{[k_1][k_3]}{[L]^2} + 1} \quad \text{(Eq. 5)}$$

The concentration of phorbol ester at which half of the PKC is translocated, that is the ED_{50} value of the translocation dose-response curve, is as follows.

$$[L_{0.5}] = \frac{-([k_3] + [k_4]) + \sqrt{([k_3] + [k_4])^2 + 4[k_1][k_3]}}{2} \qquad \text{(Eq. 6)}$$

Solving Equation 5, fitting it to the dose-response data for translocation (Fig. 2), we obtained the following apparent dissociation constants: 390 nM for k_1, 15 nM for k_2, 8.9 nM for k_3, and 230 nM for k_4, assuming that the dissociation constant of phorbol ester to each zinc finger is changed by a factor of 125 (7) upon the proline-glycine mutation. The shapes and slopes of the translocation curves as well as the ED_{50} values predicted from these parameters and Equation 5 show good agreement with the experimental data (Fig. 2) and with the empirical fit to the Hill equation (Table I).

The derived parameters for translocation assist in the conceptual understanding of aspects of our experimental findings. Because of the difference between the zinc fingers in binding affinities, the first zinc finger has little influence on the ED_{50} for translocation either in the wild type or double mutant (contributing only 3.4% to the total translocation at the ED_{50}) and negligible influence in the first zinc finger mutant (only 0.03% contribution to the total). This lack of contribution of the first zinc finger explains two otherwise paradoxical features. First, why the double mutant shows only a 138-fold shift in dose-response curve rather than a shift of 125 × 125-fold (*i.e.* 15,625-fold), which would have been expected had both zinc fingers contributed equally. Second, why the translocation fails to show strong positive cooperativity with a Hill coefficient significantly larger than 1. Consistent with our model, the one mutant showing cooperativity is that in the second zinc finger, for which the two affinities are closest to one another and an effect on binding to both zinc fingers can be manifest.

Our ongoing analysis, requiring different molecular constructs and methodologies, will hopefully provide insight into the relationship of the zinc fingers in translocation vis-a-vis other measures of PKC-ligand interaction such as enzymatic activation or ligand binding as determined directly.

The findings reported here that the second zinc finger of PKCδ suffices for PKC translocation by PMA should not be overgeneralized. The model of PKC activation that emerged from the x-ray crystallographic structure for the zinc finger-phorbol ester complex (7) was that the phorbol ester capped a hydrophilic cleft in an otherwise hydrophobic surface on the zinc finger, promoting its ability to interact with the membrane. Obviously, two features of the phorbol ester will contribute to the energy of the ternary phorbol ester-zinc finger-phospholipid complex: 1) the ability of the functional groups of the phorbol ester to form hydrogen bonds with the corresponding functional groups within the cleft, together with van der Waals interactions between hydrophobic residues of the phorbol ester and corresponding hydrophobic regions in the cleft; 2) hydrophobic interactions of the phorbol ester side chains with the membrane. This latter contribution is what makes PMA a more potent ligand than phorbol 12,13-diacetate (15), for example. Whether the combination of these contributions to stabilization of the ternary complex is sufficient to drive membrane translocation upon occupancy of the second zinc finger alone will necessarily depend on the nature and concentration of the ligand, on the degree of negative surface charge of the membrane, and on other factors that may stabilize the unfolded form of the enzyme, *i.e.* occupancy of the catalytic site. Consistent with this concept, Riedel reported that the relatively hydrophilic ligand (−)-indolactam V was only able to generate a PKC response in yeast when neither zinc finger had been deleted (10).

Our demonstration that the two zinc fingers of PKCδ are not equivalent for PKC translocation in NIH 3T3 cells agrees with the lack of equivalence reported by Riedel and co-workers using constructs of PKCα deleted in the individual zinc fingers and analyzed for their ability to modulate yeast cell growth as a function of ligand concentration (10). It likewise agrees qualitatively with the elegant study from Stubbs and co-workers (16) demonstrating biphasic binding *in vitro* to PKCα using the fluorescent phorbol ester analog sapintoxin D. In both cases, however, the substantial difference in methodologies prevents detailed comparison. Our current findings provide a framework for interpreting our earlier report, using PKCα, that we could distinguish two different interactions of phorbol esters with PKC that differed both in affinity and structure-activity (15). Under conditions limiting for membrane association, PKCα enzymatic activation required higher phorbol ester concentrations than did binding and had a greater dependence on the lipophilicity of the ligand. Although the full consequences of the asymmetry in the zinc fingers of PKC remain to be resolved, this issue appears to have great potential for the development of ligands that have novel effects on the PKC pathway.

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