Differential Roles of the Tandem C1 Domains of Protein Kinase C δ in the Biphasic Down-Regulation Induced by Bryostatin 1

Patricia S. Lorenzo, Krisztina Bögi, Kathleen M. Hughes, Maryam Beheshti, Dipak Bhattacharyya, Susan H. Garfield, George R. Pettit, and Peter M. Blumberg

ABSTRACT

Bryostatin 1 (Bryo), currently in clinical trials, has been shown to induce a biphasic concentration-response curve for down-regulating protein kinase C (PKC) δ, with protection of the enzyme from down-regulation at high Bryo doses. In our ongoing studies to identify the basis for this unique behavior of PKCδ, we examined the participation of the two ligand binding sites (C1a and C1b) in the regulatory domain of the enzyme. Three mutants of PKCδ prepared by introducing a point mutation in either C1a or C1b or both C1a and C1b were overexpressed in NIH 3T3 cells. All of the constructs retained a biphasic response to down-regulation assessed after 24-h treatment with Bryo. However, the roles of the individual C1 domains were different for the two phases of the response. For down-regulation, both the C1a and the C1b mutants displayed equivalent 3–4-fold reductions in their affinities for the ligand. For protection from down-regulation, a reduced protection was observed for the C1a mutant, which showed a broader biphasic curve compared with those for wild-type PKCδ and the C1b mutant. Like wild-type PKCδ, all of the mutants showed the same subcellular partitioning of the protected enzyme to the particulate fraction of the cells, arguing against changes in sensitivity to show the same subcellular partitioning of the protected enzyme to the particulate fraction of the cells, arguing against changes in sensitivity to down-regulation observed at high concentrations of Bryo. Thus, Bryo induces a unique biphasic down-regulation of PKCδ, with high nanomolar concentrations of Bryo protecting this isoform from down-regulation in NIH 3T3 cells (14), primary mouse keratinocytes (15), B16/F10 melanocytes (18), and HeLa cells (19). The mechanisms involved in the differences in down-regulation by Bryo and phorbol esters remain unclear.

INTRODUCTION

Bryo, the prototype of a group of naturally occurring macrocyclic lactones, was isolated from the marine organism Bugula neritina, which contains two C1 binding domains, we sought to evaluate the hypothesis that the individual C1a and C1b domains played distinct roles in down-regulation and in protection from down-regulation. In the current work, we present results obtained with PKCδ mutants in the C1a and/or C1b domains overexpressed in NIH 3T3 cells. We chose to introduce point mutations on the C1 domains that were expected to reduce the binding affinity for the ligand without disrupting the structure of the C1 domain. Our findings revealed that the C1a and C1b domains have equivalent roles in the down-regulation by Bryo, whereas a modified C1a favors a lack of protection from down-regulation at high Bryo concentrations.

MATERIALS AND METHODS

Site-directed Mutagenesis of PKCδ. Three PKCδ mutants in the C1 domain were prepared by site-directed mutagenesis, as described in detail by Szallasi et al. (21). In brief, the consensus proline in position 11 of each C1 domain of mouse PKCδ was converted into glycine by site-directed mutagenesis, simultaneously introducing a KpnI site to facilitate the selection of the mutated clones. The single mutants (C1a and C1b) and the double mutant (C1a/C1b) were then subcloned along with wild-type PKCδ into the mammalian e-epitope-tagging vector MTH (22) for expression of the constructs in NIH 3T3 cells. We have shown previously that the tagged, overexpressed PKCδ shows similar response to phorbol esters compared to the endogenous enzyme (21).
Expression of PKCδ-MTH Proteins in NIH 3T3 Cells. NIH 3T3 cells were grown in DMEM supplemented with 4500 mg/liter glucose, 4 mM l-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, and 10% fetal bovine serum. Cells were transfected with the MTH-PKCδ expression vectors using LipofectAMINE according to the procedure recommended by the manufacturer and selected for 2 weeks in medium supplemented with 750 µg/ml G418. After the selection, single colonies were picked, expanded, and screened for the presence of the different PKC proteins by Western blot analysis. Analyses were routinely carried out on transfected cell pools of each PKC construct.

Western Blot Analysis. Confluent cultures (60-mm diameter) were treated with Bryo for 24 h at 37°C. After incubation, cultures were rinsed two times with ice-cold PBS, and cells were then harvested into lysis buffer [20 mM Tris-HCl (pH 7.4), 5 mM EGTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 20 µg leupeptin], followed by sonication. Protein content was measured by a micromethod using the Bio-Rad Protein Assay. Twenty µg of lysates were mixed with equal volumes of 2× SDS sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.71 M β-mercaptoethanol, and bromphenol blue] and subjected to SDS-PAGE using 8% polyacrylamide gels, followed by electrophoresis onto nitrocellulose membranes.

For the immunostaining, we used an e-tag-specific antibody raised against COOH-terminal amino acids 726–737 of PKCδ. This antibody recognizes the e-tag present in all of the PKCs expressed from the MTH-PKCδ vector. The tagged PKCδ can be distinguished from endogenous PKCs by its difference in molecular weight. Nonspecific binding of the antibody to the membranes was blocked by a 20-min incubation with 5% dry milk in PBS. The membranes were then probed for 2 h at room temperature with 1 µg/µl anti-e-tag antibody. After the incubation, the membranes were washed for 20 min with PBS containing 0.05% Tween 20 (PBS-Tween) and then incubated for 45 min with horseradish peroxidase-conjugated antirabbit IgG. After the membranes were rinsed for 1 h in PBS-Tween, the immunostaining was visualized by enhanced chemiluminescence.

The amount of tagged PKCδ remaining 24 h after Bryo treatment was determined by densitometry of the Western blots and was expressed relative to that present in untreated cells. Apparent affinities for Bryo-induced down regulation (Kd) and protection (Kpr) were then estimated from fitting of the data to a biphasic concentration-response curve using the following equation:

\[ F(x) = A + (1 - A)[1 - x/(K_a + x)] + Bx/(K_p + x)[x/(K_a + x)] \]

where \( F(x) \) represents the fraction of PKCδ remaining as a function of Bryo concentration (x), and A and B are nonnegative numbers representing the fraction of PKCδ not subject to down-regulation and the fraction of PKCδ able to be protected by Bryo, respectively.

Cell Fractionation. After the cells were harvested and sonicated as described above, the lysates were centrifuged at 100,000 × g for 1 h at 4°C. The supernatants were collected as the soluble fraction. The pellets were sonicated again in lysis buffer and then solubilized by adding 1% Triton X-100. After a 2-h incubation at 4°C, the lysates were centrifuged at 100,000 × g for 1 h at 4°C. These supernatants were referred to as the particulate fraction.

Recombinant PKCδ Proteins. Recombinant PKC δ wild type and the C1a and C1b mutants were expressed in Sf9 cells using the baculovirus expression system. Construction of recombinant baculovirus transfer vector and expression and purification of wild-type PKCδ are described elsewhere (23). For the C1 mutants, a (His)6-tag was added to the NH2-terminus of the PKCδ proteins to facilitate the purification. In brief, the murine full-length cDNA clones of the PKCδ-C1 mutants were subcloned into the pBlue Bac His2 vector and ligated into the plasmid polylinker. This restriction site was created by ligating a phosphorylated linker containing the MluI site (New England Biolab, New England, MA) into pEGFP-N1 digested with Smal. The construct was grown in competent Escherichia coli, isolated, and verified by sequencing. To create the GFP-PKCδ fusion clone, the cDNA of the PKCδ wild type or C1 mutants was excised from a MTH-PKCδ plasmid by digestion with Xhol and MluI and ligated into the modified GFP vector using the same restriction sites. DNA sequencing of the GFP-PKCδ constructs confirmed the intended reading frame (Laboratory of Experimental Carcinogenesis, DNA Minicore, National Cancer Institute, NIH, Bethesda, MD).

Expression of GFP-PKCδ Proteins in NIH 3T3 Cells. NIH 3T3 cells were grown and maintained in the same media as described above. Twenty-four h before transfection, cells were seeded onto 40-mm round glass coverslips at a density of 5 × 10^5 cells/coverslip. Transfection with the GFP-PKCδ constructs was performed using the LipofectAMINE Plus method according to the manufacturer’s instructions. All experiments were performed 48–72 h after transfection.

Confocal fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head (Bio-Rad, Hercules, CA) mounted on a Nikon Optiphot microscope with a ×60 planapochromat lens. Excitation at 488 nm was provided by a krypton-argon gas laser with a 522/32 emission filter for green fluorescence. For living cell imaging, a Biotechnes Focht Chamber System (Biotechnes, Butler, PA) was inverted and attached to the microscope stage with a custom stage adapter. The cells plated on a 40-mm round coverslip were enclosed in the chamber and connected to a temperature controller set at 37°C, and media were perfused through the chamber with a Lambda microperfusion pump. Sequential images of the same cell were collected at various time points using LaserSharp software.

Binding of [26-3H]Bryo. Binding of [26-3H]Bryo was determined using a filtration assay as described previously (13). Briefly, saturation binding assays were performed with seven concentrations of [26-3H]Bryo (specific activity = 481 GBq/mmol), ranging from 0.5–32 nM. Partially purified PKCδ proteins were incubated with [26-3H]Bryo at 37°C for 5 min in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM MgCl2, 1 mM EGTA, and lipid micelles containing 300 µg/ml phosphatidylserine and 1.5 mg/ml Triton X-100. After incubation, samples were chilled on ice for 5 min, and two 50-µl aliquots were then removed and applied onto Whatman ion exchange paper discs (DE-81) for determination of bound ligand. After a 30-s absorption, the containing 20 mM Tris-HCl (pH 7.4) and 55% (v/v) methanol. For determination of total ligand, two additional 50-µl samples were applied onto paper discs and measured directly for radioactivity. Nonspecific binding was determined in the absence of added PKCδ protein.

Binding of [3H]PDBu. Binding of [3H]PDBu was measured using the polyethylene glycol precipitation assay as described previously (24). The assay mixture contained 50 mM Tris-HCl (pH 7.4), 1 mM MgCl2, 1 mM EGTA, and 100 µg/ml phosphatidylserine vesicles. Incubations were carried out at 18°C for 5 min. Nonspecific binding was measured using an excess of nonradioactive PDBu (30 µM).

Binding Data Analysis. Equilibrium dissociation constants (Kd) were determined from saturation curves using Origin 5.0 software (Microcal Software, Northampton, MA). All values are expressed as the mean ± SE. Comparisons were made using one-way ANOVA followed by post hoc analysis with Tukey’s test (multiple comparison) or Student’s t test [two sample comparison; GraphPad Prism 2.01 (GraphPad Software Inc., San Diego, CA)].

Materials. Bryo was isolated from B. neritina as described previously (1). DMEM was purchased from Hyclone (Logan, UT). Fetal bovine serum, LipofectAMINE, G418, and PKCδ polyclonal antibody were obtained from Life Technologies, Inc. (Gaithersburg, MD). Enhanced chemiluminescence reagents were from Amersham Life Sciences (Arlington Heights, IL), and the horseradish peroxidase-conjugated antirabbit IgG was obtained from Bio-Rad. SDS-PAGE disc gels were purchased from Novex (San Diego, CA). DE-81 Whatman ion exchange discs were obtained from Whatman Ltd. (Clifton, NJ). [3H]PDBu (777 GBq/mmol) was purchased from New England Nuclear (Boston, MA). [3H]Bryo was prepared as described previously for [3H]bryostatin 4.
Table 1  Dissociation constants (K_d) for [3H]PDBu and [26-3H]Bryo for PKCδ wild type and individual C1 mutants

Experiments were performed as described in “Materials and Methods.” Data represent the means ± SE from the number of experiments in parentheses.

<table>
<thead>
<tr>
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<th>[3H]PDBu K_d (nM)</th>
<th>[26-3H]Bryo K_d (nM)</th>
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<tbody>
<tr>
<td>PKCδ wild type</td>
<td>0.36 ± 0.05 (3)</td>
<td>0.84 ± 0.18 (2)</td>
</tr>
<tr>
<td>C1a mutant</td>
<td>0.62 ± 0.13 (3)</td>
<td>0.31 ± 0.05 (3)</td>
</tr>
<tr>
<td>C1b mutant</td>
<td>2.29 ± 0.30a (3)</td>
<td>4.54 ± 0.87a (3)</td>
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*P < 0.05 indicates a statistically significant difference compared to PKCδ wild type or C1a mutant.

RESULTS

Binding of [3H]PDBu and [3H]Bryo to PKCδ Wild Type and C1 Mutants. To explore the role of the individual C1 domains of PKCδ in the biphasic down-regulation induced by Bryo, point mutations were introduced in the first domain (C1a), the second domain (C1b), or both domains (C1a/C1b) of the enzyme. We chose to mutate consensus proline 11 in the C1 domain to glycine, based on computer modeling that suggested a loss in binding affinity without disruption of the C1 domain structure.

Proline 11 is predicted to play an important role in the folding of one of the two loops that form the binding cleft in the C1 domain. Using the isolated C1b domain, we had found that the proline-glycine point mutation induced a 200-fold reduction in the [3H]PDBu apparent binding affinity (26). We next determined the apparent binding affinity for [3H]PDBu of the intact PKCδ in response to the proline 11-to-glycine mutations in the individual C1 domains (Table 1). Either the C1a or the C1b PKCδ mutants, as well as the wild-type enzyme, showed saturation binding curves that fit better to a one-site than a two-site binding model according to statistical comparisons (GraphPad Prism 2.01; data not shown). The C1a mutant showed a slight but statistically insignificant decrease in the [3H]PDBu binding affinity compared to PKCδ wild type, whereas the C1b mutant displayed a 6-fold lower affinity. Taking into account the marked reduction in the [3H]PDBu binding affinity of the isolated, mutated C1b domain of PKCδ and the lack of a second low-affinity component in the saturation binding curves for the individual C1 mutants, it is plausible that the binding observed under our experimental conditions for the C1 mutants corresponds to interaction with the unmutated C1 domain.

Saturation analysis of [3H]Bryo binding to PKCδ wild type and individual C1 mutants also showed one-component binding curves (Fig. 1). As for [3H]PDBu binding, both PKCδ wild type and the C1a mutant displayed similar dissociation constants for [3H]Bryo binding, whereas the C1b mutant showed a lower affinity (Table 1).

It is important to note that direct comparison of the binding properties of [3H]PDBu and [3H]Bryo is not possible due to the differences in lipid environment under the distinct experimental conditions (phosphatidylserine/Triton X-100 micelles for [3H]Bryo versus phosphatidylserine vesicles for [3H]PDBu). The protocol for [3H]Bryo binding in lipid micelles was developed to overcome the methodological problems related to the high potency of Bryo, which, under lipid vesicle conditions, shows picomolar binding affinity (13). Under
Fig. 2. Effect of Bryo on the down-regulation of PKCδ wild type and C1 mutants. NIH 3T3 cells transfected with PKCδ wild type and the mutants in the C1a, C1b, and C1a/C1b domains were treated with the indicated concentrations of Bryo for 24 h. Samples from the total cell lysate were prepared for SDS-PAGE, and Western immunoblotting was performed as described in “Materials and Methods.” The amount of PKC protein was quantitated by densitometry and expressed as a percentage of the amount of protein present in the untreated cells. A: PKCδ wild type; ○, C1a mutant; and △, C1b mutant. B: PKCδ wild type; □, C1a/C1b mutant. Dose-response curves were fitted using nonlinear regression analysis. The means ± SE of six to eight experiments per group are shown.

Triton X-100 micelle conditions, the PKCδ wild type showed a 10-fold decrease in binding affinity for [3H]PDBu.3

Down-Regulation of PKCδ Wild Type and C1 Mutants Induced by Bryo. In previous studies using NIH 3T3 cells, the concentration-response curves for the down-regulation of endogenous PKCδ by Bryo were biphasic (14). We also observed biphasic down-regulation in the case of the overexpressed wild-type PKCδ as well as the C1a, C1b, and C1a/C1b mutants of PKCδ (Fig. 2, A and B). However, the mutants displayed both qualitative and quantitative differences in the patterns of response for both down-regulation and protection from down-regulation induced by Bryo.

Mutations in either the first (C1a) or the second (C1b) ligand binding domain caused a 3- to 4-fold shift in the K_{dr} induced by Bryo, and the double mutant (C1a/C1b) showed a further loss of affinity by approximately 1 order of magnitude (Table 2). This pattern of response resembled which we described previously for translocation on Bryo treatment for the same C1 mutants. For translocation, we had likewise found that both individual C1a and C1b mutants showed a reduced affinity and were equivalent and that the double mutant C1a/C1b showed a further loss in potency of Bryo (27).

In contrast to the similar effects of the C1a and C1b mutants on the down-regulation at low Bryo concentrations, the protection with high concentrations of Bryo differed for the C1a and C1b domains. This lack of equivalence between the individual C1a and C1b mutants was evident in two aspects of the response: (a) the absolute level of PKC protection; and (b) the relative difference in the potencies for down-regulation and for protection, as reflected in the spread of the biphasic dose-response curves (Fig. 2).

Mutation in the C1b domain caused a similar level of protection at high Bryo concentrations compared with wild-type PKCδ up to the maximal dose of Bryo examined (1 μM). Higher concentrations of Bryo were not examined because of concern about nonspecific toxicity due to membrane perturbation. The C1a mutant and the C1a/C1b double mutant were similar to one another and showed a reduced level of protection relative to the wild type for Bryo concentrations up to 1 μM.

K_{pr} values were calculated from the dose-response curves (Table 2). Although the exact numbers will depend on the assumptions used for fitting the data, the quantitative values mirror the qualitative conclusion that C1a plays the predominant role in protection. Mutation in C1a caused a 27-fold shift in the affinity for protection relative to the wild type, compared with only a 3-fold shift for down-regulation. In contrast, mutation in C1b caused similar 4-fold shifts for both down-regulation and protection. Similarly, the affinity for protection of the C1a/C1b double mutant reflected a 17-fold shift relative to the C1b single mutant and only a 3-fold shift relative to the C1a single mutant.

Localization of PKCδ Wild Type and C1 Mutants in Response to Bryo. We had previously reported that PKCδ protected from down-regulation by high concentrations of Bryo localized to the particulate, Triton X-100-soluble fraction of the cells (14, 20). Total lysates from cells expressing the C1 mutants as well as PKCδ wild type displayed a biphasic concentration-response curve (Fig. 3). Subcellular fractionation revealed a preferential partition of the isoenzymes protected from down-regulation into the detergent-soluble fraction of the cells (Fig. 3).

To further explore the possibility that a different pattern of translocation of the PKCδ proteins upon short-term Bryo treatment may determine a different susceptibility to down-regulation, we monitored the subcellular distribution of PKCδ and C1 mutants in living NIH 3T3 cells using GFP-tagged constructs. GFP-tagged constructs, including GFP-PKCδ, have been used by a number of groups to monitor PKC localization, and the properties of the fusion proteins have been found to be similar to those of the wild-type enzymes (28–30). When expressed in untreated cells, PKCδ wild type as well as the C1 mutants distributed relatively uniformly throughout the cell, with some localization in the perinuclear region (Fig. 4). Among the PKCδ mutants, the C1a mutant displayed a more prominent perinuclear

| Table 2 Potency of Bryo in inducing down-regulation and protection from down-regulation of PKCδ wild type and C1 mutants |
|-----------------|-----------------|-----------------|-----------------|
|                 | K_{dr} (nM)     | K_{pr} (nM)     | K_{dr}/K_{pr}   |
| PKCδ wild type  | 0.20 ± 0.03     | 6.1 ± 2.2       | 30              |
| C1a mutant      | 0.59 ± 0.10     | 165 ± 35^b      | 280             |
| C1b mutant      | 0.73 ± 0.08     | 27 ± 5^b        | 37              |
| C1a/C1b mutant  | 6.1 ± 0.6^b     | 449 ± 85^b      | 74              |

3 N. E. Lewin and P. M. Blumberg, unpublished data.

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Provided no convincing evidence that differences in localization of the contrast, the mutation in C1a diminished the extent of protection. For protection, in analyses on C1a and C1b in inducing down-regulation. For protection, in the ability of Bryo to induce down-regulation compared with the fusion protein on prolonged incubation. As with the subcellular fractionation, the analysis with the GFP-PKC constructs provided no convincing evidence that differences in localization of the PKC wild type and mutants could account for the observed differences in susceptibility to down-regulation/protection by Bryo.

**DISCUSSION**

In the present study, we examined the contributions of the C1a and C1b domains of PKCδ to the biphasic down-regulation induced by Bryo. The results demonstrate nonequivalent roles for the C1a and C1b domains in the ability of Bryo to induce down-regulation compared with protection from down-regulation. Thus, Bryo showed an equal dependence on C1a and C1b in inducing down-regulation. For protection, in contrast, the mutation in C1a diminished the extent of protection.

Binding analysis of the individual C1 mutants of PKCδ revealed a lower affinity interaction of the C1b mutant compared with the C1a mutant for both PDBu and Bryo (present results). Because of the lack of a low affinity site in the binding curves, the affinities observed for both mutants are likely to correspond to the affinities of the unmutated C1 domain present in the holoenzyme. If this premise is valid, then the intact C1b domain possesses a higher affinity for phorbol esters and Bryo than the C1a domain in the context of the PKCδ holoenzyme. However, this difference in binding affinities does not correlate with the effects of the mutation in the C1 domain of PKCδ on the down-regulation and protection induced by Bryo. Therefore, it should be emphasized that different biological end points may show different relatives roles of the two C1 domains. Several studies have suggested differences in recognition for C1a and C1b by phorbol esters and related compounds as reflected in different PKC-mediated responses. We have described a preferential role of the C1b domain for translocation of PKCδ in response to PMA and the indole alkaloids, although C1a and C1b were equivalent for translocation by mezerein or Bryo (27). Using deletion mutants of a different isoenzyme (PKCε) lacking either the C1a domain (and pseudosubstrate region) or the C1b domain (and C2), Shieh et al. (31) suggested that bryostatin 5 acted predominantly through the C1b domain, that mezerein acted predominantly through the C1a domain, and PMA acted equivalently through both domains for inhibition of doubling time in yeast. In extensive studies, Slater et al. (32) have documented, using PKCα, heterogeneous binding for various ligands as measured by inhibition of the fluorescent ligand sapintoxin D, although the correspondence to the C1a and C1b domains still remains to be established. For the observed differences between our present binding data on PKCδ wild type and mutants and the previous results on translocation, it is plausible that the lipid environment can play a role. Whereas the binding assays were performed in the presence of phosphatidylserine (present results), in the in vivo translocation experiments the lipid environment reflects the much more complex structure and composition of the cellular membranes.

One possibility that could account for the change in sensitivity of the mutants in the biphasic response to Bryo might be a difference in the subcellular localization. In this way, a different localization could change the accessibility of the isoenzyme to the down-regulation pathway. However, we found little support for this possibility using two different approaches. First, subcellular fractionation showed a similar distribution for all of the PKCδ variants examined on 24-h treatment with vehicle or various concentrations of Bryo. Second, the studies performed on living cells demonstrated a similar pattern of translocation for the GFP-tagged PKCδ wild type and C1 mutants upon short-term Bryo treatment (up to 35 min). The latter experiment should have detected more subtle changes in localization than would the subcellular fractionation.

Caspases and the proteasome are two major pathways implicated in PKCδ proteolytic degradation. PKCδ has been shown to be a substrate for caspase-3, and the catalytic fragment of the isoform has been linked to apoptosis execution (33, 34). The proteasome, on the other hand, has been implicated in phorbol ester-mediated down-regulation.
of PKCδ, PKCα, and PKCε (35, 36). PKCα and PKCε have also been shown to be down-regulated by Bryo in a proteasome-mediated manner (36, 37). In separate studies, we have begun to explore the role of these pathways in the biphasic down-regulation induced by Bryo. Preliminary results indicated that neither the caspase inhibitor Z-VAD-FMK nor the proteosome inhibitor lactacystin blocked the down-regulation of PKCδ induced by Bryo. On the other hand, both

Fig. 4. Effects of Bryo on the localization of GFP-PKCδ wild type and C1 mutants in NIH 3T3 cells. The figure illustrates sequential confocal images of NIH 3T3 transiently expressing PKCδ wild type (A), C1α mutant (B), C1b mutant (C), or C1a/C1b mutant (D). The images were taken at 0, 5, and 35 min after treatment with different concentrations of Bryo (indicated on the left of each panel). All of the experiments were performed at 37°C, as described in “Materials and Methods.” Cells shown are representative of two independent experiments. Bar, 5 mm.
inhibitors counteracted the protection of PKCδ from down-regulation induced by high concentrations of Bryo, consistent with their interference on Bryo-induced protection through an indirect mechanism. Although the basis for these effects remains undetermined, the findings described in the present work demonstrate the different participation of the two C1 domains of PKCδ in response to Bryo-induced down-regulation.

Our findings on down-regulation of PKCδ qualitatively parallel our previous observations on translocation: (a) equivalence of the C1a and C1b mutants; and (b) further loss of potency for the C1a/C1b double mutant. This result is consistent with translocation and down-regulation being coupled processes. On the other hand, it should be noted that we observed somewhat smaller differences in potencies for down-regulation than for translocation. Thus, the C1a/C1b double mutant
showed a 30-fold decrease in potency, compared to the 230-fold decrease reported earlier for translation (27). This difference may reflect, among other factors, the nonequilibrium nature of down-regulation measurements and the different incubation times (24 versus 3 h).

In contrast to down-regulation, the protection from down-regulation displayed differential dependence on C1a and C1b, with diminished affinity of C1a reducing protection. This important finding suggests that the ratio of occupancy of C1a and C1b by Bryo may influence the final outcome in the protection from down-regulation. Because other ligands, e.g., PMA, fail to protect, it is clear that unique structural aspects of Bryo are also involved. If this model is valid, design of appropriate ligands selective for the C1a domain of PKCβ might provide novel agents with Bryo-like activities.

An important finding is that both the C1a and the C1b domains influence both the high (down-regulation)- and low (protection from down-regulation)-affinity dose-response curves. This suggests that other mechanisms must contribute to the heterogeneity in binding affinities. We have previously observed large differences in structure-activity relations for phorbol ester interaction with PKC isoforms in cellular assays and in purified preparations (23, 38), likewise arguing for marked effects of modular factors. Attractive and not mutually exclusive candidates include phosphorylation, lipid environment, and interacting proteins (7, 39–41).

REFERENCES

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