Bryostatin 1 (Bryo) has been shown to induce biphasic dose-response curves for down-regulating protein kinase Cδ (PKCδ) as well as for protecting PKCδ from down-regulation induced by phorbol 12-myristate 13-acetate (PMA). To identify regions within PKCδ that confer these responses to Bryo, we utilized reciprocal PKCa and PKCδ chimeras (PKCaδ and PKCδα) constructed by exchanging the regulatory and catalytic domains of these PKCs. These chimeras and wild-type PKCaα and PKCδδ constructed in the same way were stably expressed in NIH 3T3 fibroblasts. Twenty-four h of treatment with Bryo induced a biphasic dose-response curve for down-regulating both wild-type PKCδδ and the PKCαδ chimera. In contrast, Bryo led to a nearly complete down-regulation of both PKCaα and PKCδα and also produced a faster mobility form of these species on SDS-polyacrylamide gel electrophoresis. The nature of both the regulatory and, to a lesser extent, the catalytic domains affected the potency of Bryo to down-regulate the chimeric PKC proteins as well as to protect PKCaα and PKCδα from down-regulation. Bryo at high concentrations also inhibited the down-regulation of PKCδδ and PKCαδ induced by 1 μM PMA when co-applied. The portion of PKC protected by Bryo from down-regulation by either Bryo or PMA was localized in the particulate fraction of the cells. We conclude that the catalytic domain of PKCδ confers protection from down-regulation induced by Bryo or Bryo plus PMA, suggesting that this domain contains the isotype-specific determinants involved in the unique effect of Bryo on PKCδ.

The protein kinase C (PKC) isoforms compose a large family of phospholipid-dependent serine-threonine kinases involved in cellular signaling (1–3). PKC has also been found to be the major intracellular target for the tumor-promoting phorbol esters (4, 5) as well as for the novel antineoplastic compound bryostatin 1 (Bryo) (6–9). Bryo, like the phorbol esters, binds to both the classical and novel PKC isozymes with high affinity (10), activating and subsequently down-regulating them (11, 12). Nevertheless, in contrast to the phorbol esters, Bryo is not a complete tumor promoter (13). Moreover, Bryo has been found to have antagonistic actions on certain phorbol ester-mediated effects such as the differentiation of the human promyelocytic leukemia cell line HL-60 (14), the inhibition of chemically induced differentiation of Friend erythroleukemia cells (15), and the proliferative response of human T lymphocytes (16). The mechanism(s) for the differential effects of Bryo and the phorbol esters remain(s) unclear. However, differences between PKC activation and down-regulation by phorbol esters and Bryo have been found that may contribute to the different biology. For example, Bryo was reported to be more effective compared with phorbol esters at inducing down-regulation of some PKC isoforms, such as PKCa and PKCδ in human T lymphocytes (16) and PKCs in breast cancer cell lines (17) as well as in LLC-MK2 epithelial cells (18). In addition, Bryo shows a unique pattern of down-regulation of the PKCδ isoform in intact NIH 3T3 cells (12), in B16/F10 melanoma cells (19), and in primary mouse keratinocytes (20). Moreover, Bryo has been reported to protect the PKCδ isoform from down-regulation by phorbol esters in NIH 3T3 cells (12), in primary mouse keratinocytes (20), and in rat 3Y1 fibroblasts (21). Clarifying the mechanism(s) for the unusual activity of Bryo on the PKC family is of great interest since Bryo is currently in clinical trials for several malignancies (22).

The aim of this work was to analyze the molecular basis underlying the biphasic effect of Bryo on PKCδ down-regulation in intact NIH 3T3 fibroblasts. PKC isoforms are closely related structurally and consist of a single polypeptide chain that can be functionally divided into halves comprising an N-terminal regulatory domain and a C-terminal catalytic domain connected by a flexible hinge region (1, 23). In this study, we utilized PKC chimeras prepared by exchanging the regulatory and catalytic domains of the PKCδ and PKCα isoforms, taking advantage of the high sequence homology in the hinge regions. Among the PKC isoforms naturally occurring in NIH 3T3 cells, we selected the PKCα isoform as a partner for the chimeras with PKCδ because Bryo is markedly less potent in NIH 3T3 cells for down-regulating endogenous PKCα compared with PKCδ (12). It was hoped that this difference would help to distinguish the contributions of the individual domains of each isozyme in the PKC chimeras.

Our findings implicate the catalytic domain of PKCδ in the protection elicited by Bryo from the PKCδ down-regulation by either Bryo or the phorbol ester PMA. We also observed that both the regulatory and catalytic domains of the PKCα and PKCδ chimeras affected the potency of Bryo both to down-regulate the PKC proteins and to protect the PKCs with the δ-catalytic domain from down-regulation at high Bryo doses.

**EXPERIMENTAL PROCEDURES**

**Construction of PKCα and PKCδ Chimeras—Two PKC chimeras were generated by exchanging the regulatory and catalytic domains of PKCα and PKCδ as reported by Acs et al. (24). In brief, the catalytic and regulatory domains of PKCα and PKCδ were amplified by polymerase chain reaction. Internal primers contained a sequence from the C3 region in the catalytic domain of PKC that is common to both PKCs and
PKCα. A restriction site (SpeI) was also included in the internal primers for cloning purposes. After polymerase chain reaction, the regulatory and catalytic domains were separately cloned into the pGEM-T vector, and after the first cloning procedure, the catalytic domains were subcloned into the vectors containing the regulatory domains. The SpeI site was then mutated back to the original sequence by site-directed mutagenesis. In this way, two PKC chimeras, PKCa/b and PKCa/a, along with wild-type PKCa/a and PKCa/b were generated. PKCa/b refers to the chimera with the PKCα regulatory domain and the PKCβ catalytic domain; PKCa/a refers to the reciprocal chimera. The PKC constructs were finally sonicated into the mammalian e epitope-tagging vector MTH (25) for expression of the PKC proteins in NIH 3T3 cells.

Transfection of Cells and Cell Culture—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 4500 mg/liter glucose, 4 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Advanced Biotechnologies Inc., Columbia, MD), and 10% fetal bovine serum (Life Technologies, Inc.). Cells were transfected with the expression vector using LipofectAMINE (Life Technologies, Inc.) according to the procedure recommended by the manufacturer and selected for 2 weeks in medium supplemented with 750 μg/ml G418 (Life Technologies, Inc.). After the selection, single colonies were picked, expanded, and screened for the presence of the different PKC proteins by Western blot analysis. Routinely, analyses were carried out on transfected cell pools of each PKC construct.

Western Blot Analysis—Confluent cultures (60-mm diameter) were treated with Bryo or PMA or with a combination of both agents for 24 h at 37°C. The compounds were resuspended in dimethyl sulfoxide (0.1% final concentration). After incubation, cultures were rinsed two times with ice-cold phosphate-buffered saline, and then cells were harvested in lysis buffer (20 mM Tris-HCl (pH 7.4), 5 mM EGTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 20 μg/ml leupeptin), followed by sonication. Protein content was measured by a micromethod using the bicinchoninic acid method of Lowry et al. (Bio-Rad). After the membranes were stained with specific antibodies 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 solubilized in lysis buffer containing 1% Triton X-100; they were then sonicated and centrifuged again at 100,000 × g for 1 h at 4°C. These supernatants are referred to as the “particulate” fraction.

Statistical Analysis—Results are expressed as mean ± S.E. The significance between two mean values was determined by the two-tailed Student’s t test. Differences at p < 0.05 were regarded as significant.

Materials—PMA was purchased from LC Services (Woburn, MA). Bryo was isolated as described previously (6).

RESULTS

Analysis of the Transfected NIH 3T3 Cell Clones—In a previous study, we showed that long-term Bryo treatment induces a biphasic dose-response curve for down-regulating PKCβ in NIH 3T3 fibroblasts (12). In the present study, we asked whether the dose-dependent resistance to down-regulation could be attributed to a specific domain of PKCβ. For this purpose, we utilized two PKC chimeras in which the regulatory and catalytic domains of PKCa and PKCβ were exchanged. These chimeric proteins, as well as “wild-type” PKCβ and PKCa/a constructed in the same way as the chimeras, were subcloned into the e epitope-tagging MTH mammalian expression vector and transfected into NIH 3T3 cells as described under “Experimental Procedures.” Transfected cells were tested for expression of the different PKC proteins by Western blot analysis using a specific antibody against the e epitope (Fig. 1A), and stable clones of each PKC protein were expanded for further studies. The chimeric nature of PKCa/b and PKCa/a was confirmed by immunoblotting with specific antibodies against PKCa or PKCβ (Fig. 1, B and C). We have previously determined that these chimeras retain both [3H]phorbol 12,13-dibutyrate binding and kinase activity like their endogenous PKC counterparts (24).

Down-regulation of the Reciprocal PKCa and PKCβ Chimeras by Bryo—Confluent cultures of NIH 3T3 cells were treated for 24 h with increasing concentrations of Bryo (up to 1 μM) and...
lysed, and the levels of the overexpressed PKC proteins were determined by Western blotting of total cell protein. Like wild-type PKCδ/δ (Fig. 2), the PKCa/δ chimera showed a biphasic response to Bryo-induced down-regulation. At the maximal concentration of Bryo assayed (1 μM), the amount of the PKC protein protected from down-regulation reached 40–50% of the control values for both PKCa/δ and wild-type PKCδ/δ (Fig. 2). Fractionation of the cell lysates revealed that the PKC protein still persisting in the cells at high concentrations of Bryo was localized in the particulate fraction (Fig. 3). In contrast to PKCδ/δ and PKCa/δ, PKCa/α showed the expected monophasic dose-response curve for down-regulation by Bryo; the PKCα/α chimera, like PKCa/α, also showed a monophasic curve (Fig. 2). Interestingly, in addition to inducing a nearly complete down-regulation of both PKCa/α and wild-type PKCα/α, Bryo also produced a faster mobility form of these species in a dose-dependent manner (Fig. 2).

The potency of Bryo to induce the down-regulation of the different overexpressed PKC species was calculated from the dose-response curves fitted using nonlinear regression analysis. Although the catalytic domain influenced the pattern of down-regulation, the regulatory domain affected the potency of Bryo to down-regulate the chimeric PKC proteins (Fig. 4). Thus, Bryo was significantly more potent for inducing the down-regulation of wild-type PKCδ/δ compared with the PKCa/δ chimera (ED50chimera = 0.15 ± 0.02 nM and ED50PKCa/α = 1.29 ± 0.19 nM (n = 5–6), p < 0.001), and the PKCδ/α chimera was more sensitive than wild-type PKCa/α (ED50PKCa/α = 0.07 ± 0.01 nM and ED50PKCδ/α = 2.86 ± 0.32 nM (n = 3–6), p < 0.001) to Bryo treatment (Fig. 4). The influence of the regulatory domain on the protection of PKCa/δ and PKCδ/δ from the down-regulation induced by Bryo was also observed in the dose-response curves. Thus, the maximal protection by Bryo was achieved at 100 nM for wild-type PKCδ/δ and only at 1 μM for the PKCα/δ chimera.

The catalytic domain of the PKC chimeras also somewhat affected the sensitivity to Bryo. The potency of Bryo to induce down-regulation was higher for both the PKCa/δ and PKCδ/α chimeras than for wild-type PKCa/α and PKCδ/δ, respectively (ED50PKCa/α = 1.29 ± 0.19 nM and ED50PKCδ/δ = 2.86 ± 0.32 nM (n = 3–6), p < 0.01; ED50PKCa/α = 0.07 ± 0.01 nM and ED50PKCδ/δ = 0.15 ± 0.02 nM (n = 5–6), p < 0.01).

Effects of Bryo on the PMA-induced Down-regulation of PKCa/δ and Wild-type PKCδ/δ—Bryo has been shown not only to produce a biphasic response to PKCδ down-regulation,
but also to prevent the down-regulation of this isoform induced by PMA (12). These findings prompted us to examine whether the δ-catalytic domain of the novel PKC could also be involved in the protection from the PMA-induced down-regulation elicited by Bryo.

Twenty-four h of treatment with PMA (1 nM to 1 μM) down-regulated both PKCδ/δ and PKCα/δ from the cells in a dose-dependent manner (Fig. 5). For the co-treatment with Bryo, the PMA concentration selected (1 μM) was one that produced a maximal down-regulation of both PKC species analyzed. When different concentrations of Bryo (0.1 nM to 1 μM) were co-applied with 1 μM PMA, Bryo partially prevented the down-regulation induced by PMA of both PKCα/δ and wild-type PKCδ/δ (Fig. 6). This effect was dose-dependent, with a maximum occurring at 100 nM and at 1 μM for PKCδ/δ and the PKCα/δ chimera, respectively. The reduction in the down-regulation observed for PMA in the presence of Bryo was similar to the level of down-regulation induced by high doses of Bryo alone (Figs. 4 and 6). Moreover, the PKC protected from the PMA-induced down-regulation was localized in the particulate fraction, as has been observed for the treatment with Bryo alone (Figs. 3 and 7).

**DISCUSSION**

These results show that in NIH 3T3 cells overexpressing PKCα and PKCδ chimeras, the PKCδ catalytic domain conferred the selective biphasic response to down-regulation induced by in vivo Bryo treatment. Likewise, the δ-catalytic domain also contained the determinants important for the protection elicited by Bryo from the PKCδ down-regulation by the phorbol ester PMA.

Under our experimental conditions, the potency of Bryo to induce the down-regulation of the PKCα and PKCδ chimeras was influenced by the nature of both the regulatory and, to a lesser extent, the catalytic domains of PKC. Since the regulatory domain possesses the phorbol ester pharmacophore, to which Bryo binds with very high affinity (26), it is not surprising that the regulatory domain of different isoforms of PKC, in this case PKCα or PKCδ, could determine different sensitivities to Bryo treatment. From the present results, we found a higher potency of Bryo to down-regulate the PKC proteins with the δ-regulatory domain (wild-type PKCδ/δ and the PKCδ/α chimera) compared with the proteins with the α-regulatory domain (wild-type PKCα/α and the PKCα/δ chimera). These findings are in agreement with the observation that Bryo has a higher potency to translocate and down-regulate endogenous PKCδ compared with PKCα in NIH 3T3 cells (12). The influence of the catalytic domain of PKC on the sensitivity to Bryo fits with our recent report on the modulatory role of the catalytic domain in the PMA-induced translocation of PKCα, PKCδ, and PKCδ chimeras (24). Our results here showed a higher potency of Bryo to induce the down-regulation of the PKCα/δ and PKCδ/α chimeras compared with PKCα/α and PKCδ/δ, respectively. The previous results on PKCα regulatory chimeras show greater potency of PMA to translocate the PKCα/δ and PKCα/δ chimeras compared with wild-type PKCα/α (24). Many biological effects induced by Bryo are characterized by a biphasic response and a predominance over the phorbol ester effects (27). Although the mechanisms involved remain to be elucidated, differential modulation by Bryo of the PKC isoforms appears consistent with some effects. For example, the blockade by Bryo of both the inhibition by PMA of cornified envelope formation in mouse keratinocytes (20) and the tumor promotion by PMA in rat fibroblasts overexpressing the c-src proto-oncogene (21) correlates with the protection by Bryo from...
PKCδ down-regulation. We had previously suggested that a target other than PKC might mediate the action by Bryo in protecting PKCδ from PMA-induced down-regulation since the protection is noncompetitive with the phorbol ester (20). However, if the mechanism by which Bryo protected PKCδ from down-regulation was through a target different from PKCδ, then the potency of Bryo to induce that protection should be independent of the affinity of Bryo for PKCδ. In contrast, we report here that the regulatory domain of the PKCδ catalytic chimera determined the potency of Bryo to protect PKC from down-regulation. These results suggest that the protection reflects a low affinity interaction of Bryo with PKCδ itself. This low affinity interaction might involve occupancy of the second of the two C1 zinc finger domains, PKC in a suboptimal lipid environment, or a differentially phosphorylated form of PKCδ.

Phosphorylation of PKC has been demonstrated to play a role in activation and down-regulation. For PKCβII, it has been found that phosphorylation of serine 660 regulates the proteolytic stability of the kinase (28). For PKCs, it has been reported that phosphorylation of serine 657 controls the accumulation of the active enzyme (29), and the conversion of serine 657 to alanine leads to premature down-regulation in response to PMA treatment (30). In addition, phosphorylation of threonine 638 in the catalytic domain of PKCα critically controls the inactivation of this isozyme, even though it is not required for the kinase function of PKCs per se (31). Conceivably, PKCδ also requires phosphorylation/dedephosphorylation of serine and/or threonine residues for stabilization/degradation of the competent conformation of the enzyme. By modifying the ratio between phosphorylation and dephosphorylation, Bryo might affect PKC down-regulation. Changes in the phosphorylation of PKC have often been reflected by shifts in the mobility of this protein on SDS-PAGE. Although under our experimental conditions we did not observe any shift in the electrophoretic mobility of the PKCδ catalytic chimeras, it is possible that they were simply not resolved under our conditions of SDS-PAGE. Studies are therefore ongoing to further assess this issue.

Interestingly, for the PKCs catalytic chimeras, Bryo treatment was associated with faster migrating forms on SDS-PAGE accompanying the disappearance of the slower migrating ones. It is tempting to speculate that the shift in the electrophoretic mobility resulted from a change in phosphorylation induced by Bryo. In this regard, Bryo has been reported to produce faster mobility PKCα bands on SDS-PAGE in LLC-MK² cells and human fibroblasts as a consequence of dephosphorylation, which, in turn, predisposed PKC to degradation via the proteasome pathway (18, 32).

The possibility exists that the faster mobility forms of the PKCα catalytic chimeras induced by Bryo treatment represent a state resistant to down-regulation, as is the case for the PKCδ catalytic chimeras. Nevertheless, while the δ-catalytic PKCs protected by Bryo from down-regulation localized in the Triton X-100-soluble (membrane) particulate fraction of the cell (this study), the faster migrating forms of the α-catalytic PKCs were found in the Triton X-100-insoluble (cytoskeleton) fraction (data not shown). Hence, different mechanisms are likely to contribute to those effects. For the δ-catalytic PKCs, Bryo seems to induce translocation of the chimeras, but blocks the membrane-driven process of down-regulation. Since down-regulation is generally attributed to increased proteolysis with no change in the rate of synthesis (33), Bryo could affect PKCδ proteolysis itself. Many intracellular proteases have been implicated in PKC down-regulation, such as calpain (34) and the multicatalytic proteinase complex proteasome (35). Although it remains unclear whether a particular protease makes a major contribution to the proteolytic degradation of a specific PKC isoform, it is interesting to note that PEST sequences, peptide motifs that target proteins for degradation via the proteasome (36), have been found in PKCα, PKCβ, and PKCδ, but not in PKCδ (32). This finding suggests that different proteolytic pathways might be involved in the down-regulation of different PKCs. In summary, both the phosphorylation status of PKC and the proteolytic pathway involved in its degradation could be targets for the action of Bryo on the PKCδ protection from down-regulation. Further studies are necessary to identify potential in vivo phosphorylation sites as well as proteolytic signals driving PKCδ to a particular degradation pathway in the cell.

The results presented here demonstrate a role of the δ-catalytic domain in the protection induced by Bryo from PKCδ down-regulation and represent another example in which the catalytic domain of a specific PKC isoform is involved in a selective effect. The catalytic domains of PKC have been found to play a role in several PKC isotype-specific functions. Examples include the PKCβII catalytic domain being responsible for the PKCβII isotype-specific translocation to the nucleus in K562 cells transfected with PKCα and PKCβII chimeras (37) and the catalytic domain of PKCδ in reciprocal PKCδ and PKCε chimeras mediating the phorbol ester-induced macrophage differentiation of mouse promyelocytes (38). Moreover, PKCβII (II) and PKCβII (I) showed distinct localization patterns in U937 promonocytic leukemia cells (Ref. 39; for review, see Ref. 40) even though they are splice variants that differ by only 50 amino acids in the catalytic domain (41). Characterization of the determinants in the catalytic domain of PKCδ responsible for the isotype-specific effect of Bryo could provide deeper insight not only into the mechanism of action of Bryo, but also into the elucidation of the structural basis for isotype-specific responses of PKC.

REFERENCES

1. Nishizuka, Y. (1988) Nature 334, 661–665
2. Stabel, S., and Parker, P. J. (1991) Pharmacol. Ther. 51, 71–95
3. Newton, A. C. (1995) J. Biol. Chem. 270, 28485–28498
4. Ashendel, C. L. (1985) Biochim. Biophys. Acta 822, 219–242
5. Blumberg, P. M. (1988) Cancer Res. 48, 1–8
6. Petit, G. R., Herald, C. L., Deubock, D. L., Herald, D. L., Arnold, E., and Clardy, J. (1982) J. Am. Chem. Soc. 104, 6846–6848
7. Petit, G. R. (1991) Prog. Chem. Org. Nat. Prod. 57, 153–212
8. Berkow, R. L., and Kraft, A. S. (1985) Biochem. Biophys. Res. Commun. 131, 1109–1116
9. Smith, J. B., Smith, L., and Petit, G. R. (1985) Biochem. Biophys. Res. Commun. 123, 938–945
10. Katakami, M. G., Lewin, N. E., Gao, F., Petit, G. R., and Blumberg, P. M. (1994) Mol. Pharmacol. 46, 374–379
11. Huwiler, A., Fabbro, D., and Pfeilschifter, J. (1994) Biochem. Pharmacol. 48, 689–700
12. Szalasili, Z., Smith, C. B., Petit, G. R., and Blumberg, P. M. (1994) J. Biol. Chem. 269, 2118–2124
13. Hennings, H., Blumberg, P. M., Petit, G. R., Herald, C. L., Shores, R., and Yuspa, S. H. (1987) Cancer Res. 47, 1543–1546
14. Kraft, A. S., Smith, J. B., and Berkow, R. L. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 1334–1338
15. Dell’Aquila, M. L., Nguyen, H. T., Herald, C. L., Petit, G. R., and Blumberg, P. M. (1987) Cancer Res. 47, 6006–6009
16. Isakov, N., Galron, D., Mustelin, T., Petit, G. R., and Altman, A. (1993) J. Immunol. 150, 1195–1204
17. Kennedy, M. J., Prestigiacomo, L. J., Tyler, G., May, W. S., and Davidson, N. E. (1992) Cancer Res. 52, 1278–1293
18. Lee, H.-W., Smith, L., Petit, G. R., and Smith, J. B. (1996) Am. J. Physiol. 271, C304–C311
19. Szalasili, Z., Du, L., Levine, R., Lewin, N. E., Nguyen, P. N., Williams, M. D., Petit, G. R., and Blumberg, P. M. (1996) Cancer Res. 56, 2105–2111
20. Szalasili, Z., Denning, M. F., Smith, C. B., Dlugosz, A. A., Yuspa, S. H., Petit, G. R., and Blumberg, P. M. (1994) Mol. Pharmacol. 46, 840–856
21. Lu, Z., Hornia, A., Jiang, Y.-W., Zang, Q., Ohno, S., and Fuster, D. A. (1997) Mol. Cell. Biol. 17, 3418–3428
22. Piida, J. M., Cheson, B. D., and Phillips, P. H. (1996) Oncology (Baltimore) 10, 740–742
23. Hug, H., and Sarre, T. F. (1993) Biochem. J. 291, 329–343
24. Acu, P., Bogi, K., Lorenzo, P. S., Marquez, A. M., Biro, T., Szalasili, Z., and Blumberg, P. M. (1997) J. Biol. Chem. 272, 22148–22153
25. Olah, Z., Lehel, C., Jakab, G., and Anderson, W. B. (1994) Anal. Biochem. 221, 94–102
26. Wender, P. A., Cribs, C. M., Koehler, K. F., Sharkey, N. A., Herald, C. L.,
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