Bryostatin 1 Induces Biphasic Activation of Protein Kinase D in Intact Cells

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Bryostatin 1 and phorbol esters are both potent activators of protein kinase C (PKC), although their specific biological effects can differ in many systems. Here, we report that bryostatin 1 activates protein kinase D (PKD), a novel serine/threonine protein kinase, in intact Swiss 3T3 cells and secondary mouse embryo fibroblasts and in COS-7 cells transiently transfected with a PKD expression construct. The dose response of PKD activation induced by bryostatin 1 follows a striking biphasic pattern with maximal activation achieved at a concentration of 10 nM. Higher concentrations of bryostatin 1 (100 nM) reduced PKD activation induced by phorbol 12,13-dibutyrate to levels stimulated by bryostatin 1 alone. Bryostatin 1-induced PKD activation was markedly attenuated by treatment of cells with the PKC inhibitors bisindolylmaleimide I and Ro 31-8220. However, these agents did not inhibit PKD activity when added directly to in vitro kinase assays, suggesting that bryostatin 1 stimulates PKD activation through a PKC-dependent pathway in intact cells. Our results raise the possibility that activated PKD in intact cells could mediate some of the multiple biphasic biological responses induced by bryostatin 1.

Bryostatin 1 is a natural macrocyclic lactone with potent antineoplastic properties in a variety of animal models (1–3) and has entered clinical trials as a potential therapeutic agent (4, 5). Bryostatin family members bind to and activate classic and novel isoforms of protein kinase C (PKC)1 (6–9), the major cellular targets of the tumor-promoting phorbol esters (10). Despite appearing to bind to the same cellular targets, the biological responses induced by bryostatin 1 frequently differ from those induced by phorbol esters. For example, many bryostatin 1-mediated effects have unusual characteristics such as biphasic dose-response relationships, delayed kinetics, and the ability to inhibit phorbol ester-induced responses (7, 11–16). The precise mechanism(s) by which bryostatin 1 induces these biological effects remain poorly understood.

The newly identified protein kinase D (PKD) is a mouse serine/threonine protein kinase with distinct structural features and enzymological properties (17–19). In particular, the catalytic domain of PKD, which is distantly related to Ca2+-regulated kinases, shows little homology to the highly conserved regions of the kinase domain of the PKC family. As a consequence of this, PKD does not phosphorylate a variety of known PKC substrates, indicating that PKD has a distinct substrate specificity (18, 19).

The amino-terminal region of PKD contains a putative transmembrane domain, two cysteine-rich zinc finger-like motifs, and a pleckstrin homology domain. Unlike all known PKC isoforms, PKD does not contain a pseudosubstrate motif upstream of the cysteine-rich region, and the sequence separating the cysteine-rich repeats of PKD (95 amino acids) is substantially longer than that of classical and novel PKCs (28 and 35 amino acids, respectively). Additionally, residues Ala-146, Ala-154, and Tyr-182 in the consensus cysteine-rich motif of PKD differ from those found in PKCs. However, both immunopurified PKD and a fusion protein containing the cysteine-rich region of PKD bind phorbol esters with high affinity, and PKD is directly stimulated in vitro by these agents, or by diacylglycerol, in the presence of phospholipids (18, 19). A human protein kinase called atypical PKCμ (20) with 92% homology to PKD is also stimulated in vitro by phorbol esters and phospholipids (21). These results indicate that PKD/PKCμ is phorbol ester/diacylglycerol-stimulated protein kinases. Recent studies have demonstrated a novel mechanism of activation of PKD. Specifically, treatment of intact cells with biologically active phorbol esters induces phosphorylation-dependent activation of PKD through a PKC-dependent pathway (22). PKD activity recovered from phorbol ester-stimulated cells can be measured by kinase assays in the absence of lipid activators. These results revealed an unsuspected connection between PKCs and PKD and suggested that PKD can function parallel to and/or downstream of PKC in signal transduction pathways.

The differences between the biological effects elicited by phorbol esters and bryostatin 1 in certain systems prompted us to examine whether bryostatin 1 regulates PKD activity in intact cells. Here we report that treatment with bryostatin 1 induces PKD activation in intact Swiss 3T3 cells and MEF and in COS-7 cells transfected with a PKD expression vector. A salient feature of our results is that bryostatin 1-mediated activation of PKD follows a striking biphasic dose-response relationship. PKD activation induced by treatment with PDB was inhibited by high concentrations of bryostatin 1. These results raise the possibility that PKD could mediate some of the multiple biological responses induced by bryostatin 1.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 cells were maintained in DMEM supplemented with 10% FBS in a humidified atmosphere containing 10% CO2 at 37 °C. For experimental purposes, cells were plated in 90-mm dishes at 6 × 105 cells/dish in DMEM containing 10% FBS and used after 6–8 days, when the cells were confluent and quiescent.

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COS-7 cells were plated in 90-mm dishes at 6 × 10⁴ cells/dish in DMEM containing 10% FBS and used for transfection 1 day later. Secondary cultures of MEF were seeded in 90-mm dishes at 6 × 10⁵ cells/dish in DMEM containing 10% FBS, switched down to 0.5% FBS after 3–4 days, and were used after 24 h when the cells were confluent and quiescent.

cDNA Expression Vectors and Transfection of COS-7 Cells—The PKD cdNA fragment spanning bases 125 to 3179 was inserted into the mammalian expression vector pcDNA3, as described (19). A kinase-deficient mutant (PKDKR618M) was generated by site-directed mutagenesis using the Altered Sites II in vitro mutagenesis kit (Promega) and cloned into pcDNA (pcDNA-PKDKR618M).

Exponentially growing COS-7 cells, 40–60% confluent, were transfected with the various plasmids using Lipofectin (Life Technologies, Inc.) according to the manufacturer’s instructions. Briefly, 10 μg of DNA was used for 90-mm dishes. The DNA was diluted to 1 ml with Opti-MEM I (Life Technologies, Inc.) and then mixed with Lipofectin (20 μl) diluted to 1 ml with Opti-MEM I. After 15 min, the DNA-Lipofectin complex was diluted to 5 ml with Opti-MEM I, mixed gently, and overlaid onto rinsed (once with Opti-MEM I) COS-7 cells. The cultures were incubated at 37 °C for 6 h, and the medium was then replaced with fresh Opti-MEM I containing 10% FBS. The cells were used for experimental purposes 72 h later.

Immunoprecipitation—Cultured cells were washed three times in ice-cold phosphate-buffered saline and lysed in 50 mM Tris/HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 mM α, β, γ, and δ-thrombomodulin, and 10% glycerol, followed by SDS-PAGE analysis. Treatment with Bryostatin 1 Induces PKD Activation in Intact Cells: Dose Response and Kinetics—To determine whether bryostatin 1 induces PKD activation in intact cells, quiescent cultures of Swiss 3T3 cells were treated with increasing concentrations (0.3–100 nM) of bryostatin 1 for 30 min, lysed, and the extracts immunoprecipitated with the PA-1 antiserum. The immunoprecipitates were washed three times with 0.1% SDS, resuspended in SDS-PAGE sample buffer, and analyzed by SDS-PAGE and autoradiography.

RESULTS

Bryostatin Treatment—Confuent and quiescent cultures of Swiss 3T3 cells were treated with 10 nM bryostatin 1 for 30 min and lysed. The lysates were then incubated for 30 min at 37 °C with either 1 μM microcystin or an equivalent volume of solvent. PKD was then immunoprecipitated and subjected to in vitro kinase assays, as described above.

Western Blot Analysis—For Western blot analysis of PKD, immunoprecipitates were washed three times with lysis buffer A (see above), incubated with 0.75 mg/ml of the immunizing peptide in kinase buffer for 30 min at 4 °C for 3 h with the PA-1 anti-peptide antiserum (1:100 dilution), washed four times (5 min each) in 75 mM H₃PO₄. The papers were incubated at 4 °C for 3 h at 37 °C for 10 min, the reaction terminated by washing the P-81 paper four times (5 min each) in 75 mM H₃PO₄ and then spotting 80 μl of supernatant onto P-81 phosphocellulose paper. Free [γ³²P]ATP and then washed twice in phosphate-free DMEM and incubated at 37 °C with this medium containing 200 μCi/ml carrier-free [³²P]ATP for 18 h. Cells were then stimulated with either bryostatin 1 or PDB, lysed, and immunoprecipitated with PA-1 antiserum. Samples were analyzed by SDS-PAGE and autoradiography.

Bryostatin-induced PKD autophosphorylation is dose-dependent and quiescent cultures of Swiss 3T3 cells were stimulated with 10 nM bryostatin 1 for 30 min and lysed. The lysates were then incubated for 30 min at 37 °C with either 1 μM microcystin or an equivalent volume of solvent. PKD was then immunoprecipitated and subjected to in vitro kinase assays, as described above.

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Microcystin Treatment—Confuent and quiescent cultures of Swiss 3T3 cells were treated with 10 nM bryostatin 1 for 30 min and lysed. The lysates were then incubated for 30 min at 37 °C with either 1 μM microcystin or an equivalent volume of solvent. PKD was then immunoprecipitated and subjected to in vitro kinase assays, as described above.

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cultures of secondary MEF (Fig. 2) with a maximum response occurring at 10 nM. Thus, biphasic PKD activation in response to bryostatin 1 is not restricted to immortalized cell lines.

The time course of PKD activation induced by 10 nM bryostatin 1 also contrasts with that stimulated by 200 nM PDB (Fig. 3). Although PKD activation was maximal after 10 min of PDB stimulation, bryostatin 1-mediated PKD activation was delayed, with the maximal response only occurring after treatment for 30 min.

Subsequently, we determined whether bryostatin 1-mediated PKD activation could also be demonstrated using an exogenous substrate. The synthetic peptide syntide-2 (23, 24) has been identified as an efficient substrate for the catalytic domain of PKD (18) and for the full-length PKD (19). Therefore syntide-2 was chosen as a model exogenous substrate to assay PKD activity immunoprecipitated from lysates of Swiss 3T3 cells treated with increasing concentrations of bryostatin 1 (as indicated) or 200 nM PDB. As shown in Fig. 3B, a biphasic pattern of syntide-2 phosphorylation was seen in PKD immunoprecipitates from cells stimulated with bryostatin 1, which was comparable to the biphasic pattern of PKD autophosphorylation seen previously.

Bryostatin 1, at High concentrations, Antagonizes PKD Activation in Response to PDB—The biphasic bryostatin 1 dose-response curve of PKD activation in Swiss 3T3 cells prompted us to examine whether this agent, at high concentrations, could antagonize activation of PKD by PDB. To examine this possibility confluent and quiescent cultures of Swiss 3T3 cells were treated with either 200 nM PDB or with increasing concentrations of bryostatin 1 (1, 10, or 100 nM) in the absence or presence of 200 nM PDB for 30 min. The results presented in Fig. 4 show that activation of PKD by bryostatin 1 alone follows the biphasic pattern seen previously and that PDB-induced activation of PKD was not affected by the simultaneous addition of either 1 nM or 10 nM bryostatin 1. However, PKD activation induced by PDB in the presence of 100 nM bryostatin 1 was reduced to levels seen with 100 nM bryostatin 1 alone (Fig. 4). A similar antagonism of PKD activation was seen when cells were treated with 100 nM bryostatin 1 for 30 min prior to treatment with 200 nM PDB for 10 min (data not shown). These results demonstrate that treatment with high concentrations of bryostatin 1 antagonizes PKD activation induced by PDB.

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**Fig. 3. Panel A**, time course of PKD activation by bryostatin 1 and PDB. Confluent and quiescent cultures of Swiss 3T3 cells were incubated in the presence of either 10 nM bryostatin 1 or 200 nM PDB for various times as indicated and lysed. The lysates were immunoprecipitated with PA-1 antiserum and subjected to in vitro kinase assays, SDS-PAGE, and autoradiography. Autophosphorylation of PKD was quantified by scanning densitometry, and the results shown are expressed as a percentage of the maximum response induced by bryostatin 1 or PDB and are the mean ± S.E. of three independent experiments. Representative autoradiograms are shown (inset). Panel B, bryostatin 1 induces PKD activation measured by syntide-2 phosphorylation. Confluent and quiescent cultures of Swiss 3T3 cells were incubated in the presence of either increasing concentrations of bryostatin 1 for 30 min (solid bars), 200 nM PDB for 10 min (hatched bar), or an equivalent volume of solvent (open bar), lysed, and the lysates immunoprecipitated with PA-1 antiserum. PKD activity was measured in a syntide-2 phosphorylation assay as described under “Experimental Procedures.” Results shown are expressed as the fold increase over control and are the mean ± S.E. of two independent experiments, each performed in duplicate.

**Fig. 4. Effect of bryostatin 1 on PDB-induced PKD activation.** Confluent and quiescent cultures of Swiss 3T3 cells were treated with various concentrations of bryostatin 1 for 30 min in the presence or absence of 200 nM PDB as indicated, lysed, and immunoprecipitated with PA-1 antiserum. Samples were then analyzed by in vitro kinase assays, SDS-PAGE, autoradiography, and scanning densitometry. The results shown are expressed as the percentage of PKD activation induced by PDB treatment alone and are the mean ± S.E. of three independent experiments. A representative autoradiogram is shown.

**Transfected with a PKD Expression Construct**—To confirm that the bryostatin 1-induced kinase activity measured was the result of activation of PKD rather than the presence of a coprecipitating protein kinase, we examined PKD autophosphorylation in COS-7 cells transfected with either a wild type PKD expression vector (pcDNA3-PKD) or with a kinase-defective PKD mutant (pcDNA3-PKD618M) in which lysine 618 in the ATP binding site is replaced by methionine. Cells were treated with bryostatin 1 or PDB, lysed, and the immunoprecipitates subjected to in vitro kinase assays and to immunoblotting with the PA-1 antiserum. Bryostatin 1 treatment of COS-7 cells, like PDB, resulted in activation of wild type PKD (Fig. 5A, upper panel, left). In contrast, no inducible kinase activity was seen in COS-7 cells transfected with pcDNA3-PKD618M (Fig. 5A, upper panel, right) despite similar PKD and PKDK618M expression levels (Fig. 5A, lower panels). Similarly, no kinase activity was detected in COS-7 cells transfected with the control vector pcDNA3 (data not shown). These results verified that the bryostatin 1-induced kinase activity measured in PKD immunoprecipitates was caused by the activation of PKD.

**Bryostatin 1 Induces PKD Phosphorylation in Intact Swiss 3T3 Cells**—The preceding experiments demonstrated that treatment with bryostatin 1 markedly increased the level of PKD autophosphorylation in “in vitro” kinase assays. We next examined whether bryostatin 1 induces PKD phosphorylation in intact cells. Confluent and quiescent cultures of Swiss 3T3 cells metabolically labeled with 32P1 were stimulated with 10 nM bryostatin 1 for 30 min or 200 nM PDB for 10 min. Cells were lysed, immunoprecipitated with PA-1 antiserum, and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 5B, upper panel, 10 nM bryostatin 1 induced a 4.6-fold increase in the incorporation of 32P1 into PKD, which was comparable to that induced by 200 nM PDB. Interestingly, when cells were stimulated with 100 nM bryostatin 1, incorporation of 32P1 into PKD was 47% lower than after treatment of cells with 10 nM bryostatin 1 (Fig. 5B, lower panel). Thus, bryostatin 1 induces biphase phosphorylation of PKD in intact cells.

To determine further the role of phosphorylation in maintaining the activated state of PKD, we examined whether endogenous protein phosphatases could reverse bryostatin 1-induced PKD activation. Lysates of Swiss 3T3 cells treated with bryostatin 1 were incubated at 37 °C for 30 min in the absence or presence of 1 μM microcystin, a potent inhibitor of protein phosphatases 1 and 2A, prior to PKD immunoprecipitation. As shown in Fig. 5C, incubation of the lysate in the absence of microcystin resulted in a marked decrease of PKD activity. Addition of this protein phosphatase inhibitor preserved substantial PKD activity (Fig. 5C).

**Inhibitors of PKC Attenuate PKD Activation Induced by Bryostatin 1**—We next examined potential signaling pathways leading to bryostatin 1-induced PKD activation in Swiss 3T3 cells. Inhibition of a variety of kinases, including p70S6K with...
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FIG. 5. Panel A, the kinase activity induced by bryostatin 1 in PA-1 immunocomplexes is PKD rather than a coimmunoprecipitating kinase. COS-7 cells were transfected with PKD expression vectors, either pcDNA3-PKD or pcDNA3-PKD618M, as detailed under “Experimental Procedures.” After 72 h, cultures were treated with 50 nM bryostatin 1 for 30 min (Bryo) or 200 nM PDB for 10 min, lysed, and PKD immunoprecipitated as described previously. Upper panels, PKD activity was determined by in vitro kinase assays followed by SDS-PAGE, autoradiography, and scanning densitometry (IVK). Lower panels, PKD and PKD(K618M) expression levels were determined by SDS-PAGE and Western blotting (W.BLOT). Results shown are representative of three independent experiments. Panel B, bryostatin 1 induces PKD phosphorylation in vivo. Confluent and quiescent Swiss 3T3 cells were incubated for 18 h with carrier-free [32P] in phosphate-free medium and then stimulated with either 10 nM bryostatin 1 for 30 min or 200 nM PDB for 10 min (upper panel). In other experiments, [32P]Pi-labeled cells were stimulated with either 10 or 100 nM bryostatin 1 for 30 min (lower panel). Cells were lysed, immunoprecipitated with PKA-1 antisera, and the samples analyzed by SDS-PAGE and autoradiography. Results shown are representative of three independent experiments. Panel C, the protein phosphatase inhibitor microcystin prevents inactivation of PKD. Confluent and quiescent Swiss 3T3 cells were incubated with (+) or without (−) 10 nM bryostatin 1 for 30 min and lysed. Lysates were then either treated with 1 μM microcystin (M) or an equivalent volume of solvent for 30 min at 37°C. PKD was then immunoprecipitated and subjected to autophosphorylation, SDS-PAGE, and autoradiography. Results shown are representative of three independent experiments.

rapamycin (20 nM), PI 3-kinase with wortmannin (50 nM), PKA with H-89 (60 μM), and P42MAPK/P44MAPK with the selective MEK-1 inhibitor PD 098059 (10 μM) did not affect PKD activation in response to bryostatin 1 (Fig. 6). Similarly, disruption of the actin cytoskeleton and inhibition of p125FAK tyrosine phosphorylation using cytochalasin D had no effect on subsequent PKD activation induced by bryostatin 1 (Fig. 6). However, treatment of Swiss 3T3 cells with GF I or Ro 31-8220, potent inhibitors of classic and novel isoforms of PKC (25, 26), did attenuate PKD activation induced by bryostatin 1 (Fig. 6).

We therefore investigated further the role of bryostatin 1-sensitive PKCs in the activation of PKD induced by treatment of intact cells with bryostatin 1. As shown in Fig. 7A, upper panels, pretreatment of Swiss 3T3 cells with increasing concentrations of either GF I or Ro 31-8220 led to a marked dose-dependent reduction in the subsequent activation of PKD elicited by bryostatin 1. In addition, we verified that GF I and Ro 31-8220 could also prevent PKD activation induced by PDB, in agreement with previous results (22). In striking contrast neither GF I nor Ro 31-8220 inhibited PKD activity, induced by treatment of intact cells with either bryostatin 1 or PDB, when added directly to in vitro kinase assays at concentrations identical to those used in intact cells (Fig. 7A, lower panels).

To verify that GF I does not prevent PKD stimulation induced by bryostatin 1 in vitro, cultures of COS-7 cells transfected with pcDNA3-PKD were lysed, and PKD was immunoprecipitated with either 10 nM bryostatin 1 for 30 min or 200 nM PDB for 10 min, lysed, and PKD immunoprecipitated as described previously. Upper panels, PKD activity was determined by in vitro kinase assays followed by SDS-PAGE, autoradiography, and scanning densitometry (IVK). Lower panels, PKD and PKD(K618M) expression levels were determined by SDS-PAGE and Western blotting (W.BLOT). Results shown are representative of three independent experiments. Panel B, bryostatin 1 induces PKD phosphorylation in vivo. Confluent and quiescent Swiss 3T3 cells were incubated for 18 h with carrier-free [32P] in phosphate-free medium and then stimulated with either 10 nM bryostatin 1 for 30 min or 200 nM PDB for 10 min (upper panel). In other experiments, [32P]Pi-labeled cells were stimulated with either 10 or 100 nM bryostatin 1 for 30 min (lower panel). Cells were lysed, immunoprecipitated with PKA-1 antisera, and the samples analyzed by SDS-PAGE and autoradiography. Results shown are representative of two independent experiments. Panel C, the protein phosphatase inhibitor microcystin prevents inactivation of PKD. Confluent and quiescent Swiss 3T3 cells were incubated with (+) or without (−) 10 nM bryostatin 1 for 30 min and lysed. Lysates were then either treated with 1 μM microcystin (M) or an equivalent volume of solvent for 30 min at 37°C. PKD was then immunoprecipitated and subjected to autophosphorylation, SDS-PAGE, and autoradiography. Results shown are representative of three independent experiments.

FIG. 6. Bryostatin 1-induced PKD activation is attenuated by the PKC inhibitors GF I and Ro 31-8220. Confluent and quiescent cultures of Swiss 3T3 cells were treated with 3.5 μM GF I (GF), 2.5 μM Ro 31-8220 (Ro), 10 μM PD 098059 (PD), 20 nM rapamycin (RAP), 50 nM wortmannin (WOR), 60 μM H-89, 2.5 μM cyclohexalin D (CYT D), or an equivalent volume of solvent for 1 h. Cells were then incubated in the absence (open bars) or presence of 10 nM bryostatin 1 (solid bars) for 30 min. The cultures were lysed, immunoprecipitated with PA-1 antisera and PKD, subjected to in vitro kinase assays, SDS-PAGE, autoradiography, and scanning densitometry. Results shown are representative of two independent experiments and are expressed as a percentage of the maximal increase in PKD activity induced by bryostatin 1.

discussion

Bryostatin 1 binds to and activates PKC, but its biological effects differ greatly from other PKC activators such as the tumor-promoting phorbol esters (7, 11–15). These unexplained differences prompted us to examine the regulation of PKD activity by bryostatin 1 in intact cells.

The results presented here demonstrate, for the first time, that bryostatin 1 induces a striking activation of PKD in intact cultures of Swiss 3T3 cells, MEF, and in COS-7 cells transiently transfected with a PKD expression construct. Stimulation of intact cells with bryostatin 1 leads to the persistent activation of PKD which can be detected by in vitro kinase assays (as seen by autophosphorylation and syntide-2 phosphorylation) in the absence of any further lipid activators. Since the activated state of PKD induced by bryostatin 1 in intact cells is maintained during cell lysis and immunoprecipitation, it is likely that the enzyme is activated by a covalent modification. Here we demonstrate that bryostatin 1 stimulation of intact Swiss 3T3 cells markedly enhanced the incorporation of [32P] into PKD in metabolically labeled cells. The role of phosphorylation in the regulation of PKD was also suggested by treatment of bryostatin 1-stimulated Swiss 3T3 cell lysates with the protein phosphatase 1 and 2A inhibitor microcystin, which partially prevented the inactivation of PKD by endogenous protein phosphatases.
The active state induced by bryostatin 1 stimulation of intact cells could be produced by an activating autophosphorylation step or by trans-phosphorylation mediated by an upstream protein kinase, for example, bryostatin 1-sensitive PKCs. In- deed, the active state induced by bryostatin 1 stimulation of intact cells could be produced by an activating autophosphorylation step or by trans-phosphorylation mediated by an upstream protein kinase. 

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