Communication

Ubiquitination of Protein Kinase C-α and Degradation by the Proteasome*

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Bryostatins and phorbol esters acutely activate and subsequently down-regulate protein kinase C (PKC) by inducing its proteolysis via an unknown pathway. Here we show that treatment of renal epithelial cells with bryostatin 1 (Bryo) produced novel PKC-α species, which were larger than the native protein (80 kDa). The >80 kDa PKC-α species contained Ubi as indicated by immunostaining and accumulated in the presence of lactacystin, a selective inhibitor of proteolysis by the proteasome. In vitro experiments with 32P-labeled ubiquitin and membranes from Bryo-treated cells showed that PKC-α became ubiquitinated by a reaction that depended on ATP and a cytosolic fraction. Lactacystin or a peptidyl aldehyde, Bz-Gly-Leu-Ala-leucinal, which inhibits certain proteinase activities of the proteasome, inhibited Bryo-evoked disappearance of PKC-α protein from the cells. Lacta preserved Bryo-induced 32P-labeled PKC-α indicating that the proteasome inhibitor spared activated enzyme from down-regulation in vivo. These findings show that Bryo induces the degradation of PKC-α by the ubiquitin-proteasome complex.

Protein kinase C (PKC) is a large family of enzymes, many of which depend on diacylglycerol for activity (1–4). Diacylglycerol binds with a high affinity to the Cys-rich, zinc finger domains of PKC, which recruits it to the plasma membrane and turns on its kinase function (1–4). PKC is the predominant cellular receptor for bryostatins (5–7) and phorbol ester tumor promoters (2, 3, 8), which share a common pharmacophore with diacylglycerol (9). Bryostatin 1 (Bryo), like phorbol 12-myristate 13-acetate (PMA), acutely activates PKC; however, chronic exposure of mammalian cells to Bryo or PMA down-regulates PKC activity and protein (2, 3, 10–12). A dramatic increase in PKC degradation with no change in its synthesis causes the down-regulation (13). Interestingly, Bryo elicits a subset of the cellular responses evoked by PMA and antagonizes those responses it does not induce (7, 14, 15). More efficient down-regulation of PKC by Bryo compared to PMA at least partly explains PKC antagonism by Bryo (10–12).

Recently, we reported that Bryo concomitantly produced autophosphorylated, active PKC-α and a nonphosphorylated, inactive form of the kinase in renal epithelial cells (16). The nonphosphorylated form has an apparent molecular mass of 76 kDa on SDS gels compared to the 80-kDa autophosphorylated, active form (16). PKC-α is known to become catalytically competent upon phosphorylation at trans sites (Thr-495 and possibly Thr-497) by an unidentified "PKC kinase" (1, 17, 18). Removal of permissive phosphorylation from purified, recombinant PKC-βII (19) and PKC-α (20) by phosphatase treatment renders the kinase incompetent and increases its electrophoretic mobility on SDS gels from 80 to 76 kDa. Production of the 76-kDa form by Bryo or PMA in the epithelial cells was independent of protein synthesis, and pulse-chase 35S-labeling experiments indicated that the 76-kDa form was produced by dephosphorylation of activated, membrane-bound kinase (16). Greater production of the 76-kDa form at least partially explained the more rapid and efficient down-regulation of PKC-α by Bryo versus PMA (16).

Because the pathway of PKC degradation is unknown, there is little understanding of what predisposes it to degradation. Previous studies have implicated Ca2+-activated neutral proteases (calpain) or increased membrane trafficking and multiple proteases in PKC down-regulation (21–23). Here we show that treatment of renal epithelial cells with Bryo produced novel PKC-α species which were larger than the native enzyme and accumulated in the presence of lactacystin (Lacta), a highly selective inhibitor of the proteasome (24). The larger than native PKC-α species immunostained for ubiquitin (Ubi). The 26 S proteasome is the major lysosomal proteolytic pathway that depends on ATP and ubiquitination, which occurs outside the proteasome (25, 26). Ubi is activated by ATP to a high energy thiol ester intermediate by Ubi-activating enzyme (E1). Ubi-conjugating enzyme (E2) transfers activated Ubi from E1 to the protein substrate which is usually bound to a Ubi-protein ligase (E3) (25). Lacta inhibits three distinct peptidase activities of the 20 S proteolytic core of the 26 S mammalian proteasome, apparently by covalent modification of the highly conserved amino-terminal Thr of subunit IX (also called MB1) (24). Lacta preserved Bryo-evoked 32P-labeled PKC-α in vivo.

In vitro experiments with 125I-Ubi and membranes from Bryo-treated cells showed that PKC-α became ubiquitinated by a reaction that depended on ATP and a cytosolic fraction.

EXPERIMENTAL PROCEDURES

The LLC-MK2 line of renal epithelial cells from rhesus monkey (ATCC CCL 7.2) was grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% (v/v) fetal bovine serum (27). In vitro Ubiquitination of PKC-α—The ubiquitination reaction (0.2 ml) contained 75 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 3 mM ATP, 10 mM creatine phosphate, 10 μg of creatine phosphokinase, 3 mM DTT, 1 mg/ml saponin, 0.6 mg of membranes, 0.4 mg of cytosol, and 10 μM 125I-Ubi (4 × 105 cpm). Membranes and PKC-depleted cytosol were

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Prepared as described below and used as a source of PKC-α and ubiquitin-activating enzymes, respectively. Similar extents of PKC-α ubiquitination were observed in the absence and presence of soybean which was added to permeabilize the membranes. The reaction was studied by adding 0.1 ml of 95 °C 10 mM Tris-HCl, pH 7.5, containing 3% SDS, and incubation at 100 °C for 5 min. Following the addition of 0.9 ml of ice-cold buffer A, PKC-α was immunoprecipitated with 3 μg of rabbit polyclonal PKC-α antibody for 1.5 h followed by the addition of protein A-agarose for 1 h. Buffer A contained (in mM): 10 Tris-HCl, pH 7.5, 5 EDTA, 50 NaCl, 30 sodium pyrophosphate, 50 NaF, 0.1 sodium orthovanadate, 1% Triton X-100, and 0.1% Nonidet P-40. In some experiments, 3 μg of PKC-α antibody was incubated for 2 h in 50 μl of PBS with 30 μg of the PKC-α immunogen (residues 651–672) to block the antigen-binding sites. Immunoprecipitates were washed three times with ice-cold buffer A, solubilized with SDS sample solution, and fractionated by SDS-PAGE (7% gels). The gels were stained with Coomassie Blue and dried, and 125I was quantified by phosphorescence imaging (GS-250 Molecular Imager, Bio-Rad) and counted. The molecular mass standards (Bio-Rad) were serum albumin, phosphorylase B, β-galactosidase, and myosin. The positions and molecular masses (kDa) of the standards are indicated on the images of the gels.

Preparation of Membranes, Cytosol, and 125I-Ubi—Confluent cultures (10-cm diameter) were detached by trypsinization (27), washed, suspended with 1 ml of conditioned medium, and incubated at 4 °C for 1 h. After centrifugation, washed twice with 20 ml of PBS, suspended with 5 ml of ice-cold buffer B, and disrupted by 50 strokes with a Dounce homogenizer. Buffer B contained 20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 10 μM leupeptin, 10 μM aprotinin, and 2 mM DTT. Membranes were pelletted by centrifugation (100,000 g for 30 min), resuspended again with 5 ml ice-cold buffer B, centrifuged, and suspended in buffer B at ~50 μg of protein/ml.

Prior to the preparation of cytosol, 0.1 μM Bryo was added to the plating medium (40 cultures, 10-cm diameter) for 48 h to deplete PKC-α. The cells were rinsed twice with PBS, detached by scraping with PBS, collected by centrifugation, suspended with 1 ml of ice-cold 20 mM Tris-HCl, pH 7.5, containing 2 mM DTT, and disrupted by 50 strokes with a Dounce homogenizer. Particulate material was removed by centrifugation at 100,000 g for 60 min. Protein concentration was measured by the Bradford method with bovine serum albumin as a standard (Bio-Rad). 125I-Ubi was prepared by incubating bovine erythrocyte Ubi (0.5 mg) with 5 μCi of Na22 and three iOODEAHS (Fierce) for 15 min at room temperature in 0.2 ml of 0.1 M sodium phosphate buffer, pH 6.5. 125I-Ubi was separated from excess Na22 and unreacted 125I-urea by gel filtration chromatography and migrated as a single band of the appropriate molecular mass by SDS-PAGE.

PKC-α immunoprecipitation and Western Analysis—When the cultures (60-mm diameter) became confluent, the volume of the medium was reduced 1 ml to 2 ml, and fresh Bryo, lacta. β-Gly-Leu-Leu-leucinal (zGLALal), or βzGly-Leu-Ala-Leu-leucinal (zGLALol) were added as indicated from thousand-fold concentrated solutions in dimethyl sulfoxide. The cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2, and extracted with ice-cold lysis buffer as described (16). Lysis buffer contained 1% (w/v) Triton X-100 and (in mM): 10 Tris-HCl, pH 7.4, 5 EDTA, 1 phenylmethylsulfonyl fluoride, 0.1 Na3VO4, 30 sodium pyrophosphate, 50 NaF, 10 μM leupeptin, and 10 μM aprotinin. Lysate samples were preclared by incubation with 20 μl of protein A/G agorase at 4 °C for 1 h and incubated with the monoclonal antibody to rat brain PKC-α and 30 μl of protein A/G agarase at 4 °C for 3 h. Immunocomplexes were washed, and proteins were extracted with SDS sample solution as described (16). SDS-PAGE (10% gels), transfer to a PVDF membranes, and immunostaining with anti-rabbit antibody to PKC-α was done as described (16).

Western Analysis of Ubiquitinated Proteins—PKC-α was immunoprecipitated with the monoclonal antibody, separated by SDS-PAGE (10% gels), and transferred to a nitrocellulose membrane. Membranes were autodained for 20 min, incubated for 10 min with TBS and then for 1 h with blocking solution (TBS containing 0.5% dry milk), rinsed twice (5 min each) with TTBTS (TBS containing 0.05% (v/v) Tween 20), and incubated for 1 h in TTBTS containing 0.1% dry milk and a thousandfold dilution of a monoclonal Ubi antibody (4F3 asces fluid (28). TBS contained (per liter): 8 g of NaCl, 0.2 g of KCl, 3 g of Tris base, and was adjusted to pH 7.4 with HCl. Membranes were rinsed with TTBTS for 15 min, replacing the solution at 5-min intervals, and incubated for 1 h with TTBTS containing 0.1% dry milk and a 1:20,000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Transduction Laboratories). After rinsing three times with TTBTS (5 min each), immunocomplexes were visualized by incubation with LumiGLO (Eberhard and Perry Laboratories and Konica PPB film. After immunostaining for Ubi, membranes were rinsed for 24 h with TBS and immunostained for PKC-α as described previously (16).

125I [32P]PKC-α Labeling—Confluent cultures (60-mm diameter) were incubated twice with phosphate-free DMEM and incubated with 2 ml of phosphate-free DMEM containing [32P]orthophosphate for 2 h. Lacta (50 μm) was added to the labeling medium as indicated. One h later, Bryo was added to 1 μM as indicated. After 1 or 8 h, the cultures were rinsed 8 times with ice-cold PBS and extracted with 0.5 ml of ice-cold lysis buffer. Immunoprecipitation and Western analysis of PKC-α were done as described previously (16). After immunostaining for PKC-α, the membrane was rinsed extensively with TBS and autoradiographed at ~70 °C to detect [32P]-labeled PKC-α.

Materials—Ascites fluid (4F3) containing the Ubi antibody was generously provided by Dr. Linda A. Guarino (Texas A & M University, College Station, TX). A monoconal (lgG3) to an immunogen corresponding to positions 270–427 of rat brain PKC-α was from Transduction Laboratories. Affinity-purified, rabbit polyclonal IgG that specifically recognizes PKC-α (epitope residues 653–672 of rabbit PKC-α) and the peptide immunogen were from Santa Cruz Biotechnology. Lacta was from Dr. E. J. Corey (Harvard University). Bryo was isolated from Bugula neritina as described (29). zGLALal and zGLALol were synthesized as described (30, 31). Ubi from bovine erythrocytes was from Fluka. [32P]Orthophosphoric acid (9,000 Ci/mmol) and carrier-free Na22 (17 Ci/mg) were from DuPont NEN.

RESULTS AND DISCUSSION

In Vitro Ubiquitination of PKC-α—When membranes from Bryo-treated cells were incubated with 125I-Ubi in the presence of cytosol and ATP, there was a time-dependent labeling of several SDS gel bands, which were immunoprecipitated with affinity-purified polyclonal antibodies that specifically recognized the α isofrom of PKC (Fig. 1). Labeling was abolished by blocking the antigen-binding sites with the peptide immunogen (Fig. 2A). Immunoprecipitation of PKC-α from the reaction mixture with a monoclonal antibody to the hinge region of the kinase produced a similar labeling pattern as the polyclonal antibody, and blockade of the monoclonal with purified recombinant PKC-α abolished the labeling.2 Addition of excess unlabeled Ubi to the reaction mixture also abolished the labeling indicating that it was caused by ubiquitination (Fig. 2A). There were 125I-labeled bands with apparent molecular masses of approximately 90, 110, 120, and 180 kDa (Figs. 1 and 2). The 90-kDa band is the approximate mass expected for PKC-α conjugated to one or two Ubi. The >90-kDa bands probably contain multiple Ubi per kinase. Ubiquitination of PKC-α reached a peak at 2 h and decreased from 2 to 4 h (Fig. 1). The decrease may be caused by degradation by the proteasome. Ubiquitination of PKC-α depended on the presence of cytosol and ATP or ATPγS (Fig. 2), which is known to support Ubi activation by E1 (32). Cytosol contains E1, E2, and E3 enzymes (25, 26) and was prepared from cells that were incubated with 0.1 μM Bryo for 48 h to deplete PKC-α as shown by Western analysis (Fig. 2B).

Interestingly, membranes from cells that were not treated with Bryo failed to support PKC-α ubiquitination (Fig. 2A, lane 8). These membranes contained somewhat more 80-kDa PKC-α than those from cells treated with 1 μM Bryo for 4 h (Fig. 2B). The membranes from untreated cells, however, lacked 76-kDa, nonphosphorylated PKC-α which is prominent in membranes from Bryo-treated cells (Fig. 2B) as previously reported (16). These findings demonstrate ubiquitination of PKC-α in vitro and are consistent with the idea that 76-kDa PKC-α is an intermediate in the degradation pathway (16).

Lacta or zGLALal Preserves PKC-α Protein from Down-regulation in Vivo—If the proteasome is responsible for PKC down-regulation, then proteasome inhibitors would be expected to prevent its degradation. In this study, we have focused on the effect of lactacystin, a specific inhibitor of the proteasome (39), on the degradation of PKC-α. Lacta is a proteasome inhibitor that has been shown to prevent the degradation of PKC-α in vitro (16). We have also shown that lactacystin inhibits the degradation of PKC-α in vivo (17).

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FIG. 1. Dependence of PKC-α ubiquitination on time and ATP. Membranes (0.6 mg) from cells treated with 1 μM Bryo for 4 h were incubated with [35S]Ubi (40 million cpm, 10 μM) and a PKC-depleted cytosolic fraction (0.4 mg) in the presence and absence of ATP for the indicated interval. PKC-α was immunoprecipitated, fractionated by SDS-PAGE, and [35S]-Ubi was detected by phosphorescence imaging. The graph shows the [35S] content of gel slices containing the 90-kDa band, as determined by γ counting (mean ± S.E., 3 experiments).

FIG. 2. Dependence of PKC-α ubiquitination on cytosol and membranes from Bryo-treated cells. For A, membranes (0.6 mg) from cells treated with 1 μM Bryo for 4 h were incubated with [35S]Ubi (40 million cpm, 10 μM) and a PKC-depleted cytosolic fraction (0.4 mg) in the presence and absence of ATP for 2 h. PKC-α was immunoprecipitated, fractionated by SDS-PAGE, and [35S]-Ubi was detected by phosphorescence imaging. For lane 2, the PKC-α antibody was blocked with the PKC-α peptide immunogen; lane 3, the reaction mixture contained 5 mM ATP•S instead of ATP and the regenerating system; lane 4, ATP and the regenerating system were omitted from the reaction; lane 5, no membranes; lane 6, no cytosol; lane 7, 0.1 mM unlabeled Ubi was added to the reaction; and lane 8, membranes (0.6 mg) were from untreated cells. B shows Western analysis of PKC-α of the membranes (Membr.) and cytosol (Cyt.) fractions used in A. B indicates that the membranes or cytosol was from Bryo-treated cells. Filled and unfilled circles indicate 80- and 76-kDa PKC-α bands, respectively. Data are representative of at least 3 experiments.

Ubiquitination of PKC-α in Vivo—PKC-α was immunoprecipitated, and the Western blot was overexposed to detect the >80 kDa PKC-α bands, which obscured the decrease in 80-kDa PKC-α produced by Bryo (Fig. 3B). Incubation with Bryo for 8 h produced PKC-α bands with apparent molecular masses of 90 and 110 kDa (Fig. 3B). Interestingly, Lacta potentiated the Bryo-induced accumulation of the 90- and 110-kDa bands (Fig. 3B). The 90-kDa PKC-α band was observed after a 1-h Bryo treatment (Fig. 3D), but Lacta had no effect on the amount of the 90-kDa PKC-α produced by a 1-h incubation with Bryo (Fig. 3D). This finding is consistent with the idea that Lacta preserves 90-kDa PKC-α from degradation rather than increasing its production. Lacta by itself produced no 90-kDa PKC-α at 8 h (Fig. 3B) or 1 h.²

To determine whether the >80-kDa bands contained Ubi, PKC-α was immunoprecipitated from cells that were incubated for 12 h in the presence or absence of Bryo plus Lacta. Immunostaining with the 4F3 monoclonal antibody indicated that the 90- and 110-kDa PKC-α bands were ubiquitinated (Fig. 3C). In addition, there was a smear of immunostaining from 116 to 200 kDa, as would be expected for polyubiquitinated PKC-α species containing progressively more Ubi per PKC-α (Fig. 3C). There was no detectable Ubi in PKC-α immunoprecipitated from the cells that were not treated with Bryo and Lacta (Fig. 3C). Neither the 76- nor the 80-kDa PKC-α bands immunostained for Ubi, which confirms the specificity of Ubi immunostaining (Fig. 3C). The 110-kDa band was the most prominent ubiquitinated PKC-α band (Fig. 3C). The relative intensities of the 90- and 110-kDa bands suggest that the former contains more PKC-α and less Ubi than the latter (Fig. 3C).

Lacta Protects 32P-Labeled PKC-α Produced by Bryo—Deter-
[32P]orthophosphate-labeled cells were incubated for 8 h with Lacta and Bryo, the amount of [32P]-labeled PKC-a increased markedly compared to treatment with Bryo alone (Fig. 3B). Lacta had no effect on the amount of [32P]-labeled PKC-a produced by a 1-h incubation with Bryo (Fig. 3D). These data show that Lacta principally affected the disappearance of [32P]-labeled PKC-a rather than its formation. The inhibition of the disappearance of PKC-a protein by Lacta accounts, at least in part, for the increase in [32P]-labeled enzyme. [32P] was not detected in the 76- or 90-kDa PKC-a bands after either an 8- or 1-h incubation with Bryo in the absence or presence of Lacta (Fig. 3, B and D).

Previously we postulated that dephosphorylated, incompetent 76-kDa PKC-a is an intermediate in the pathway of down-regulation-induced Bryo and PMA (16). The lack of detectable [32P] in 90-kDa, ubiquitinated PKC-a is consistent with the idea that it is produced from the nonphosphorylated 76-kDa form rather than the autophosphorylated 80-kDa form. According to this hypothesis, nonphosphorylated, incompetent kinase would be a better substrate for ubiquitination than autophosphorylated PKC-a. The roles of specific phosphorylations in the ubiquitination and down-regulation of PKC-a remain to be clarified.

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