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Bryostatin 1 and Phorbol Ester Down-Modulate Protein Kinase C-α and -ϵ via the Ubiquitin/Proteasome Pathway in Human Fibroblasts

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SUMMARY

We evaluated the possibility that distinct proteolytic pathways contribute to the down-regulation of a novel (ϵ) or conventional (α) isoform of protein kinase C (PKC) in nonimmortalized human fibroblasts. Inhibitors of calpains and other cysteine proteinases, vesicle trafficking, or lysosomal proteolysis did not affect the down-regulation of PKC-α or -ϵ produced by bryostatin 1 (Bryo). Lactacystin (Lacta) and certain terminal aldehyde tripeptides or tetrapeptides, which selectively inhibit the proteasome, preserved substantial PKC-α and -ϵ protein from down-regulation by Bryo or phorbol-12-myristate-13-acetate. Lacta preserved active kinase *in vivo*, as shown by the retention of Bryo-induced autophosphorylated PKC-α. Concomitant with down-regulation, Bryo produced PKC-α and -ϵ species that were larger than the native proteins (80 and 90 kDa, respectively). Western blot analysis showed that the larger PKC-α species were ubiquitinylated. Treatment with Bryo plus Lacta synergistically increased multiubiquitinylated PKC-α, as expected if Bryo induces ubiquitinylation of PKC-α and Lacta blocks its degradation. Bryo also produced a 76-kDa, nonphosphorylated form of PKC-α and an 86-kDa form of PKC-ϵ. Phosphatase inhibitors decreased production of 76- and 86-kDa PKC-α and -ϵ by Bryo and preserved 80- and 90-kDa PKC-α and -ϵ, respectively. Our results suggest that the down-modulation of PKC-α and -ϵ occurs principally via the ubiquitin/proteasome pathway. Dephosphorylation seems to predispose PKC to ubiquitinylation.

Phorbol esters and bryostatins acutely activate and subsequently down-modulate conventional (α, β, and δ) and novel (δ, ϵ, η, and θ) isoforms of PKC in mammalian cells (1–5). The regulatory domain of conventional isoforms differs from that of novel ones, which lack a putative Ca^{2+}-binding C2 region (1, 2). Bryo, phorbol esters, and DAG, an endogenous PKC activator, bind to the two cysteine-rich, zinc finger motifs in conventional and novel isoforms (1, 2, 6). One zinc finger motif binds the activators with an affinity order of Bryo > PMA > DAG, whereas the other has the inverse affinity order (7, 8). Interestingly, in contrast to PMA, Bryo is not a carcinogen or a complete tumor promoter (9). Bryo elicits some of the same acute cellular responses as PMA but antagonizes chronic responses provoked by PMA (9–14). Faster and more effective down-regulation of PKC by Bryo compared with PMA seems to explain the antagonism of PKC (5, 13, 14). A striking increase in the degradation of PKC causes down-regulation, which can occur with no change in PKC synthesis (1, 2, 15).

Recently, we reported that Bryo induced multiubiquitinylation of PKC-α *in vitro* and in a renal epithelial cell line (16). *In vitro* ubiquitinylation of PKC-α required ATP (or ATPδS), membranes containing the 76-kDa, nonphosphorylated form of PKC, and a cytosol fraction (16). Cytosol contains Ub-activating (E1), -conjugating (E2), and -ligating (E3) enzymes (17). The ~26S proteasome is a predominantly nuclear and cytoplasmic organelle that degrades multiubiquitinylated proteins by an ATP-dependent mechanism (17). The proteasome degrades many short-lived proteins and proteins whose degradation is triggered by external stimuli (18). The novel antibiotic Lacta specifically modifies the amino-terminal threonine of subunit X of the mammalian proteasome and inhibits its three distinct peptidase activities (19). Experi-

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ABBREVIATIONS: PKC, protein kinase C; AcLLMal, *N*-acetyl-Leu-Leu-methional; AcLLNal, *N*-acetyl-Leu-Leu-norleucinal; BFA, brefeldin A; Bryo, bryostatin 1; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; Lacta, lactacystin; LB, lysis buffer; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate; TBS, Tris-buffered salt solution; TRH, thyrotropin-releasing hormone; Ub, ubiquitin; ZGLALal, benzyloxycarbonyl-Gly-Leu-Ala-leucinal; ZGLALol, benzyloxycarbonyl-Gly-Leu-Ala-leucinol.

ments with [^{3}H]Lacta, Neuro-2a cells, and brain homogenates identified proteasome subunits as the essentially exclusive cellular target of Lacta (19). Lacta spared PKC-α in renal epithelial cells from down-modulation by Bryo (16).

Some studies have implicated increased vesicle trafficking including lysosomal endocytosis and a general degradative process in PKC down-regulation (20, 21). Others have invoked calpains, Ca^{2+}-activated, cysteine proteinases, in down-modulation (22, 23). Down-regulation of PKC-ϵ seems to depend on the calpain/calpastatin system (22), whereas the Ub/proteasome pathway contributes to the down-regulation of PKC-α (16). Therefore, we tested the possibility that calpains, lysosomal proteinases, vesicle trafficking, and the Ub/proteasome pathway contribute to the degradation of a conventional (α) and a novel (ϵ) isoform of PKC in human fibroblasts. Our findings suggest that the Ub/proteasome pathway is mainly responsible for the disappearance of PKC-α and -ϵ isoforms provoked by PMA or Bryo. We also observed that decreasing the production of dephosphorylated PKC-α and -ϵ with phosphatase inhibitors antagonized down-regulation. Dephosphorylation of activated PKC seems to predispose it to ubiquitinylation, which targets it to the proteasome.

Experimental Procedures

Primary cultures of human dermal fibroblasts were initiated from forearm biopsies and grown in DMEM containing 10% fetal bovine serum as described previously (24).

Western blot analysis of PKC-α and -ϵ. Confluent cultures (35-mm diameter) were incubated at 37° in 1 ml of the plating medium in a humidified atmosphere of 95% air/5% CO_2 with the indicated additions. Compounds such as Bryo, Lacta, E64d (*N*[*N*-L-*trans*-carboxyoxiran-2-carbonyl-L-leucyl]agmatine), and peptides were dissolved in dimethylsulfoxide and added to the cultures from thousand-fold-concentrated solutions. Dimethylsulfoxide did not affect Bryo-evoked disappearance of PKC-α or -ϵ proteins or their amounts in untreated cells. Cultures were rinsed three times with ice-cold phosphate-buffered saline, 0.1 ml of ice-cold LB [1% (w/v) Triton X-100, 10 mM Tris·HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 50 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin] was added, and the cells were removed with a squeegee.

Lysates were passed through a 26-gauge needle three times and centrifuged for 20 min at 4° at 16,000 $\times$ *g*. Protein was measured according to the BCA method (Pierce Chemical, Rockford, IL) with bovine serum albumin as standard. Proteins were fractionated by SDS-PAGE (7% or 10% gels with a 1:25 ratio of *N*,*N*′-methylene-bisacrylamide/acrylamide) for 3.5 hr at 150 V at 4° to improve the resolution of faster and slower PKC-α and -ϵ bands (14). Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) at 22 V for 16–20 hr at 4°. Transfer buffer contained 25 mM Tris, 192 mM glycine, and 0.05% SDS (w/v) and was diluted 20% with methanol.

Membranes were blocked for 1 hr with TBS containing 0.5% nonfat dry milk, rinsed twice (5 min each) with TTBS [TBS containing 0.05% (v/v) Tween 20], and incubated for 1 hr in TTBS containing 0.1% dry milk and a 1:1000 dilution of an affinity purified, polyclonal antibody to PKC-α or -ϵ. TBS contained 8 g/liter NaCl, 0.2 g/liter KCl, and 3 g/liter Tris base and was adjusted to pH 7.4 with HCl. Membranes were then rinsed three times (5 min each) with TTBS and incubated for 1 hr with TTBS containing 0.1% dry milk and a 1:10,000 dilution of affinity isolated goat anti-rabbit IgG conjugated to horseradish peroxidase (Biosource International, Camarillo, CA). After rinsing three times with TTBS (5 min each), immunostaining was visualized with LumiGLO (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and Konica PPB film. Autoradiograms are representative of three or more experiments.

Immunoprecipitation of PKC. The volume of the culture medium was reduced to 2 ml (60-mm diameter) or 4 ml (100-mm diameter), and the indicated compounds were added from thousand-fold-concentrated stock solutions. The cultures were incubated for the indicated interval and extracted with ice-cold LB. Protein was measured according to the BCA method, and a sample was precleared with 20 μl of protein A/G agarose at 4° for 1 hr and incubated with a mouse monoclonal antibody to rat brain PKC-α or rat PKC-ϵ (Transduction Laboratories, Lexington, KY) and 30 μl of protein A/G agarose at 4° for 3 hr. Immunocomplexes were washed, and proteins were extracted with SDS and fractionated by SDS-PAGE, and PKC-α or -ϵ was visualized by Western blot analysis (14).

Western blot analysis of ubiquitinylated PKC-α. Cultures were incubated with the indicated additions and extracted with LB. PKC-α was immunoprecipitated with the monoclonal antibody, separated by SDS-PAGE, and electrophoretically transferred to Hybond ECL nitrocellulose (Amersham Life Science, Arlington Heights, IL). Blots were autoclaved in water for 30 min at 120° to denature Ub, incubated for 10 min with TBS, blocked for 1 hr with TBS containing 0.5% dry milk, rinsed twice (5 min each) with TTBS, and incubated for 1 hr in TTBS containing 0.1% dry milk and a 1:1000 dilution of a monoclonal Ub antibody (4F3 ascites fluid) or 2 μg/ml concentration each of protein A-purified monoclonal antibodies 1B3 and 2C5 to bovine erythrocyte Ub coupled to keyhole limpet hemocyanin (Pan-Vera, Madison, WI). Membranes were rinsed with TTBS for 15 min, with the solution replaced at 5-min intervals, and incubated for 1 hr with TTBS containing 0.1% dry milk and a 1:20,000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY). After being rinsed three times with TTBS (5 min each), ubiquitinylated proteins were visualized with LumiGLO (Kirkegaard & Perry Laboratories) and Konica PPB film. Membranes were rinsed overnight at room temperature with TBS (Fig. 5B) or stripped for 30 min at 65° with 62.5 mM Tris-Cl, pH 6.8, containing 2% SDS and 0.1 M β-mercaptoethanol (Fig. 6), and PKC-α was visualized by Western blot analysis.

^{32}P-PKC-α labeling. Confluent cultures (60-mm diameter) were rinsed twice with phosphate-free DMEM and incubated with 2 ml of phosphate-free DMEM containing 1 mCi of [^{32}P]orthophosphate for 3 hr. Bryo and/or Lacta was added as indicated, and 8 hr later the cultures were rinsed eight times with ice-cold phosphate-buffered saline and extracted with 0.5 ml of ice-cold LB. PKC-α was immunoprecipitated and visualized by Western blot analysis. After the membrane was rinsed with TBS, it was autoradiographed at −70°.

PKC activity. Confluent cultures (100-mm diameter) were incubated with the indicated additions for 20 hr in the plating medium. Cultures were rinsed, and lysates were prepared as described previously (14). PKC from three cultures was partially purified by DEAE cellulose chromatography. Fractions were assayed for PKC activity as described previously (14).

Materials. 4F3 ascites fluid was generously provided by Dr. Linda A. Guarino (Texas A & M University, College Station, TX). monoclonal antibodies to an immunogen corresponding to positions 270–427 of rat brain PKC-α or an amino-terminal fragment (residues 1–175) of rat PKC-ϵ were from Transduction Laboratories. Affinity-purified rabbit polyclonal antibodies to an epitope corresponding to amino acids 651–672 of human PKC-α or residues 723–737 of human PKC-ϵ were from Santa Cruz Biochemicals (Santa Cruz, CA). AcLL-Nal and AcLLMal were from Bachem Bioscience (King of Prussia, PA). Ub, E64d, and BFA were from Sigma Chemical (St. Louis, MO), and monensin was from Calbiochem (San Diego, CA). Lacta was obtained from Dr. E. J. Corey (Harvard University, Boston, MA). ZGLALal and ZGLALol were provided by Dr. Alexander Vinitsky (Mt. Sinai School of Medicine, City University of New York, New York, NY). Bryo was isolated from *Bugula neritina* as described previously (25).

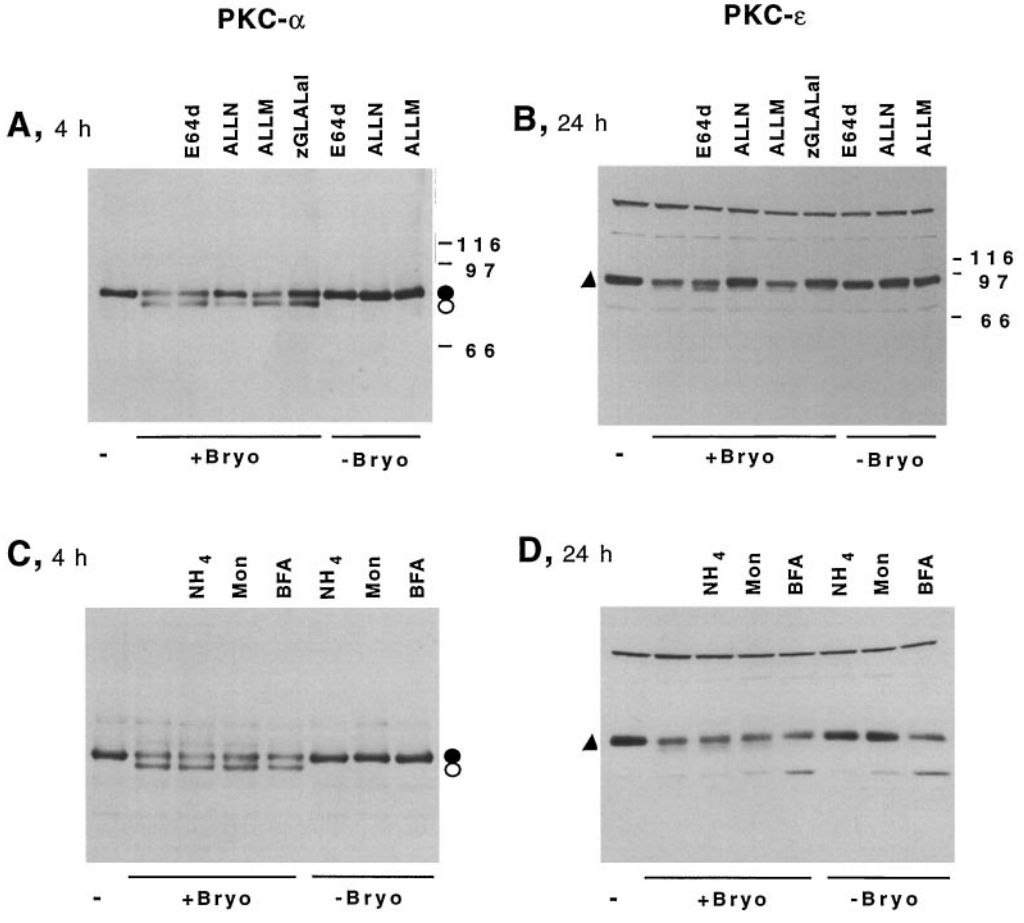

Fig. 1. Lack of effect of inhibitors of calpains, lysosomal proteinases, or vesicle trafficking on the down-regulation of PKC-α and -ε. A and B, Cultures were incubated for 1 hr with 0.1 mM of the indicated compound before adding 1 μM Bryo for 4 hr (A) or 24 hr (B). C and D, Cultures were incubated for 1 hr with 20 mM NH_4Cl, 10 μM monensin, or 0.1 μg/ml BFA before the addition of 1 μM Bryo for 4 (C) or 24 (D) hr. Cultures were extracted with LB, and proteins (A, 10 μg; B, 30 μg; C, 10 μg; D, 20 μg) were fractionated by SDS-PAGE (7% gel). PKC-α and -ε were visualized by Western blot analysis. Molecular mass markers are indicated (kDa). ● and ○, 80- and 76-kDa PKC-α bands, respectively. ▲, 90-kDa PKC-ε band.

Results

Down-modulation of PKC-α and -ε and production of faster mobility species. Human fibroblasts were incubated with 1 μM Bryo for 4 or 24 hr, and PKC was quantified by Western blot analysis. PKC-α or -ε from untreated cells migrated as a single band with an apparent molecular mass of 80 and 90 kDa, respectively (Fig. 1, A and B). Bryo provoked the disappearance of PKC-α and -ε (Fig. 1), but PKC-α disappeared much faster than the ε isoform. For example, the decrease in PKC-α produced by the 4-hr Bryo treatment was similar to that produced by the 24-hr treatment for PKC-ε (Fig. 1). The 24-hr Bryo treatment decreased PKC-α and -ε to 5 ± 2% and 36 ± 3% of control, respectively (five experiments). An 8-hr treatment with 1 μM Bryo decreased 80- and 90-kDa PKC-α and -ε bands to 22 ± 6% and 75 ± 8% of control, respectively (three experiments). In addition to depleting the 80- and 90-kDa PKC species, Bryo produced faster mobility forms of PKC-α and -ε with apparent molecular masses of ~76 and ~86 kDa, respectively (Fig. 2A). Although the 86-kDa PKC-ε band was observed in the Bryo-treated but not the control cells in all experiments, some gels (see Figs. 2A and 4C) resolved the 86- and 90-kDa bands better than others (Figs. 1B and 2B). To readily observe the 86-kDa PKC-ε band without overexposure of the slower band in untreated cells, it was necessary to apply ≥30 μg to the SDS gel (Figs. 1 and 2). The faster mobility PKC-ε band was clearly produced by a 2-hr treatment with 1 μM Bryo.[1] The specific immunoreactivity of the PKC-α and -ε species was demonstrated by sequentially immunostaining of the same membrane for each isoform and observation that both PKC-α and PKC-ε bands had distinct electrophoretic mobilities (Fig. 2A). These observations show that production of the faster mobility PKC-ε band accompanied the disappearance of the slower one, as previously shown for PKC-α (14, 16). The faster mobility PKC-α band is a nonphosphorylated species produced from active kinase in epithelial cells and fibroblasts (14, 16, 26).

Lack of effect of inhibitors of calpains, lysosomal proteolysis, and vesicle trafficking on PKC-α and -ε down-regulation. The cell-permeant cysteine protease inhibitor E64d (27) did not affect the disappearance of PKC-α and -ε evoked by Bryo (Fig. 1, A and B). Also, AcLLMal (calpain inhibitor II), which is a potent inhibitor of calpain and lysosomal cysteine proteinases such as cathepsin B (28), did not affect the disappearance of PKC-α and -ε (Fig. 1, A and B). Interestingly, AcLLNal (calpain inhibitor I) partially inhibited the down-regulation of PKC-α and -ε by Bryo (Fig. 1). AcLLNal inhibits calpain and cathepsin B with similar potencies as AcLLMal (28), but AcLLNal is ~40 times more potent than AcLLMal as an inhibitor of proteasomal peptidase activities (28). These findings suggest that the down-modulation of PKC-α and -ε depends on the 26S proteasome. Another peptidyl aldehyde, ZGLALal, which potently inhibits the proteasome (29), preserved PKC-α and -ε proteins from down-regulation by Bryo (Fig. 1) as described below. Neither NH_4Cl nor the Na^+/H^+ antiporter monensin affected

[1] Lee, H.-W., and Smith, J. B., unpublished observations.

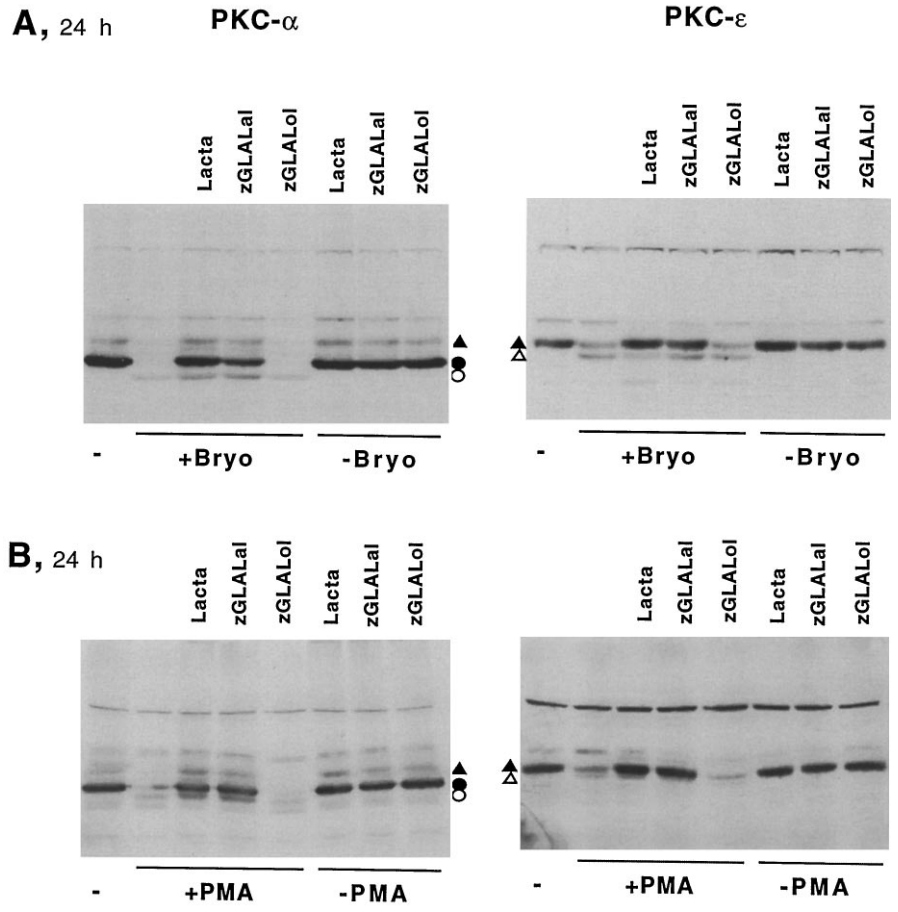

Fig. 2. Proteasome inhibitors preserve PKC-α and -ϵ proteins from down-regulation by Bryo or PMA. Cultures were incubated for 1 hr with 50 □μM Lacta, ZGLALal, or ZGLALol before the addition of 1 μM Bryo (A) or 1 μM PMA (B). At 24 hr later, they were extracted with LB, and proteins (A, 40 μg; B, 30 μg) were fractionated by SDS-PAGE (7% gel). PKC-ϵ was visualized by Western blot analysis. The membranes were washed overnight with TBS and immunostained for PKC-α. ● and ○, 80- and 76-kDa PKC-α bands, respectively. ▲ and △, 90- and 86-kDa PKC-ϵ bands, respectively. Note that 90 kDa PKC-ϵ (▲) is visible on the PKC-α blot because PKC-ϵ antibodies were not stripped from the membrane before immunostaining PKC-α.

Bryo-induced down-modulation of either PKC isoform (Fig. 1, C and D). Monensin or NH_4Cl neutralizes lysosomal acidity and inhibits lysosomal proteolysis. In addition, monensin blocks vesicle trafficking, as does BFA, which reversibly disrupts the Golgi apparatus (30, 31). Neither monensin nor BFA affected the down-regulation of PKC-α or -ϵ (Fig. 1, C and D). None of the compounds tested affected cell morphology, as observed by phase-contrast microscopy,[1] and only the 24-hr treatment with BFA, which somewhat decreased PKC-ϵ, affected PKC in cells that were not treated with Bryo (Fig. 1D). These findings support the idea that the proteasome is principally responsible for the down-regulation of a conventional and a novel PKC isoform.

Proteasome inhibitors preserve PKC-α and -ϵ from down-regulation. Lacta, which is a highly selective inhibitor of proteolysis by the proteasome (19), preserved substantial PKC-α and -ϵ from down-modulation by 1 μM Bryo or PMA (Fig. 2). Peptidyl aldehydes, such as ZGLALal, selectively inhibit proteolytic activities of the 20 S proteasome *in vitro* and 26 S-mediated intracellular degradation of ubiquitinylated proteins (29). Furthermore, ZGLALal preserved PKC-α and -ϵ in a manner similar to that of Lacta from down-regulation by Bryo (Fig. 2). The corresponding peptidyl alcohol, ZGLALol, did not affect the down-regulation of either PKC isoform (Fig. 2), as expected because ZGLALol is inactive as a proteasome inhibitor (29). Neither Lacta nor the peptides affected PKC-α or -ϵ in cells that were not treated with Bryo (Fig. 2).

Proteasome inhibitors spare PKC activity from down-regulation by low concentrations of PMA or Bryo. We used 1 μM Bryo or PMA for the experiments described above to markedly down-regulate PKC-α in 4 hr. To determine whether proteasome inhibitors preserve PKC from down-regulation evoked by prolonged incubations with low concentrations of the PKC activators, we incubated human fibroblasts with 50 nM Bryo or 0.1 μM PMA for 20 hr, which strongly down-modulated PKC-α protein and total PKC activity (Fig. 3). Lacta protected PKC-α protein and Ca^{2+} and lipid-dependent histone kinase activity from down-modulation by Bryo or PMA (Fig. 3). Cells treated with Bryo or PMA in the presence of Lacta retained 7- and 14-fold more PKC activity, respectively, than those incubated with Bryo or PMA alone (Fig. 3). For this experiment, PKC was purified by DEAE cellulose chromatography and assayed as the difference in histone kinase activity with or without Ca^{2+}, DAG, and phosphatidyl serine (14). None of the treatments affected the amount of protein extracted from the cells or eluted from the DEAE columns or the histone kinase activity measured without Ca^{2+} and lipids, which was only 3–7% of that in their presence.

Lacta preserves autophosphorylated PKC-α *in vivo*. Human fibroblasts were labeled with ^{32}P-orthophosphate for 2 hr and treated with 1 μM Bryo and/or 50 μM Lacta in the labeling medium for 8 hr (Fig. 4A). Lacta strongly preserved Bryo-induced ^{32}P-labeled PKC-α (Fig. 4A). Lacta alone did not increase ^{32}P-labeled PKC-α at 8 hr (Fig. 4A) or 1 hr (26). A 1-hr incubation with 1 μM Bryo maximally increased ^{32}P-labeled PKC-α, which subsequently decreased as PKC-α protein disappeared from the cells (26). Bisindolylmaleimide (2 μM), which selectively inhibits PKC, markedly decreased

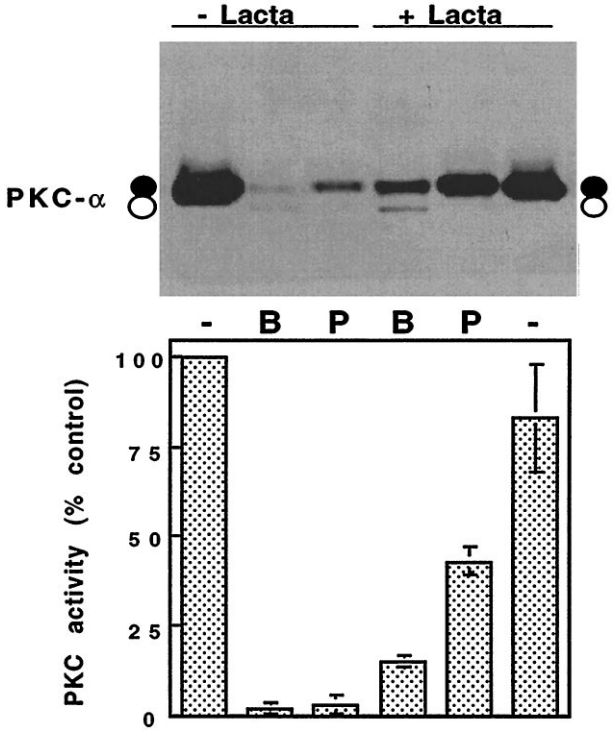

Fig. 3. Proteasome inhibitors preserve PKC activity from down-regulation by PMA or Bryo. The indicated cultures were incubated with 20 μM Lacta for 1 before the addition of 50 nM Bryo or 0.1 μM PMA. After 20 hr, PKC was extracted with Triton X-100 and partially purified by DEAE-cellulose chromatography as described previously (14). Fractions were assayed for PKC activity and total protein. A sample (6 μg) of the first column fraction, which contained most of the PKC activity, was fractionated by SDS-PAGE (10% gel), and PKC-α was visualized by Western blot analysis. PKC activities are expressed as total activity divided by total protein eluted from the DEAE cellulose column and are mean ± standard error (three experiments). PKC activity was 4–5 nmol of phosphate incorporated (μg of protein × 10 min)$^{-1}$ in the first fraction from control cells. ● and ○, 80- and 76-kDa PKC-α bands, respectively.

Bryo-induced ^{32}P-labeling of PKC-α (26), as expected for autophosphorylation. The 76-kDa PKC-α band produced by Bryo lacked detectable ^{32}P (Fig. 4A), as expected (14, 16).

Phosphatase inhibitors decrease production of the faster mobility PKC-α and -ε and inhibit down-regulation. Orthovanadate, a nonspecific phosphatase inhibitor, decreased Bryo-induced production of 76 kDa at 2, 4, and 8 hr and decreased the disappearance of 80-kDa PKC-α at 4 and 8 hr (Fig. 4B). Okadaic acid, which selectively inhibits phosphatases PP1 and PP2A (32), strikingly decreased production of 86-kDa PKC-ε by Bryo at 4 hr[1] or 16 hr and preserved 90-kDa PKC-ε at 16 hr (Fig. 4C). Okadaic acid slightly inhibited the production of 76-kDa PKC-α and the disappearance of 80-kDa PKC-α by Bryo.[1]

Production of >80-kDa ubiquitinylated PKC-α by Bryo. PKC-α was immunoprecipitated from cell lysates to readily detect >80-kDa species produced by Bryo (Fig. 5). The addition of 1–50 μM Lacta strikingly preserved ≥80-kDa PKC-α from down-regulation by Bryo (Fig. 5A). Protection of PKC-α from down-modulation was significant at 1 μM Lacta and maximal at 20 μM (Fig. 5A). The Lacta concentration dependence for the preservation of PKC-α *in vivo* is similar to that for the inhibition of proteasomal peptidase activities *in vitro* (19). Lacta by itself did not affect PKC-α protein and did not produce the >80-kDa species (Fig. 5A).

Fig. 5B shows that the 4F3 antibody, which specifically recognizes Ub (33), immunostained a ladder of bands, which were immunoprecipitated with the PKC-α antibody from cells treated with Bryo and Lacta for 12 hr. The ubiquitinylated proteins had apparent molecular masses of ~90, ~110, ~120 (doublet), and ~180 (smear) kDa (Fig. 5B). The Ub and PKC-α antibodies immunostained the 90- and 100-kDa bands, showing that they are ubiquitinylated PKC-α. Note the reciprocal intensities of 90- and 110-kDa bands immunostained by the two antibodies (Fig. 5B). A greater stoichiometry of Ub per PKC-α presumably explains the darker staining of the 110-kDa band by the Ub antibody and lighter staining by the PKC-α antibody relative to the 90-kDa band. The 90-kDa band is probably monoubiquitinylated or diubiquitinylated PKC, and the larger bands contain multiple Ub molecules per PKC-α. No ubiquitinylated bands were detected in PKC-α immunoprecipitated from untreated cells (Fig. 5B). The 80- and 76-kDa PKC-α bands from the Lacta- and Bryo-treated cells, like the 80-kDa band from untreated cells, lacked detectable Ub immunostaining (Fig. 5B).

Fig. 5, C and D, shows the time courses of the production of ubiquitinylated PKC-α by Bryo in the presence and absence of a proteasome inhibitor, Lacta or zGLALal. Significantly, Bryo alone produced ubiquitinylated PKC-α at 1 or 3 hr (Fig. 5, C and D). Ubiquitinylated PKC-α disappeared concomitantly with the disappearance of the 80- and 76-kDa forms of the kinase (Fig. 5, C and D). Lacta or ZGLALal preserved the Bryo-produced ubiquitinylated PKC-α at 8 and 24 hr, which were not detectable in cells treated with Bryo alone (Fig. 5, C and D).

Synergistic production of multiubiquitinylated PKC-α by Bryo plus Lacta. The immunostaining of ubiquitinylated PKC-α was confirmed with a combination of two monoclonal antibodies (1B3 and 2C5) that recognize different Ub epitopes. After a 4-hr incubation with Bryo and/or Lacta, the cells were lysed in the presence of 5 mM *N*-ethylmaleimide, which inactivates deubiquitinylating enzymes (34). PKC-α was immunoprecipitated and subjected to Western blot analysis with the 1B3 and 2C5 antibodies. The Ub antibodies primarily immunostained an ~180-kDa band (Fig. 6A). Bryo plus Lacta synergistically produced 180-kDa PKC-α, as shown by immunostaining with Ub or PKC-α antibodies (Fig. 6A). Bryo or Lacta alone did not markedly increase the 180-kDa PKC-α (Fig. 6A). The addition of purified Ub during the incubation with the 1B3 and 2C5 immunoglobulins abolished immunostaining of the 180-kDa PKC-α (Fig. 6A). After immunostaining with the Ub antibody, the membrane was stripped, and PKC-α bands were immunolocalized. The PKC-α antibody recognized multiple >80-kDa species, including the 180-kDa band (Fig. 6A). The 1B3 and 2C5 immunoglobulins were unable to detect shorter Ub chains, which is in contrast to the 4F3 antibodies (Figs. 5B and 6A), which recognized both short and multi-Ub chains (Fig. 5B). To maximize the amount of PKC-α immunoprecipitated, a large amount of lysate was used relative to the amount of immunoprecipitating antibody. This explains the apparent lack of disappearance of the 80-kDa band in the Bryo-treated cells (Fig. 6A). The data shown in Figs. 5 and 6 show that Bryo induces ubiquitinylation of PKC-α and that

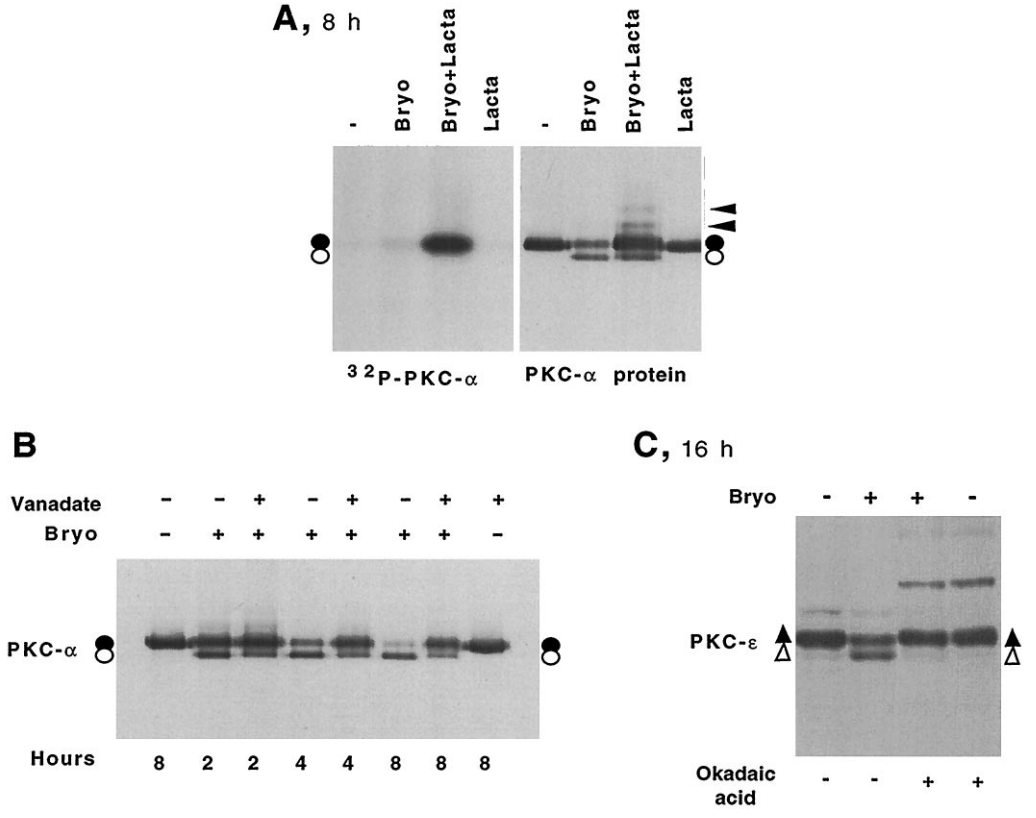

Fig. 4. Lacta preserves Bryo-induced ^{32}P-labeled PKC-α, and phosphatase inhibitors decrease production of 76-kDa PKC-α and 86-kDa PKC-ϵ. A, Cultures were incubated in phosphate-free DMEM with 1 mCi of [^{32}P]orthophosphate for 2 hr before the addition of 50 μM Lacta. At 1 hr later, 1 μM Bryo was added, and the incubation was continued for 8 hr. PKC-α was extracted with LB and immunoprecipitated from 0.24 mg of protein with 2.5 μg of monoclonal antibody. Proteins were separated by SDS-PAGE (10% gel). PKC-α was visualized by Western blot analysis, and the membrane was autoradiographed to detect ^{32}P. *Arrowheads*, ubiquitinylated PKC-α bands. ● and ○, 80- and 76-kDa PKC-α bands, respectively. B, Cultures were incubated for 1 hr with 5 mM sodium orthovanadate before the addition of 1 μM Bryo as indicated. After the indicated interval, proteins were extracted with LB, and PKC-α was immunoprecipitated from 0.16 mg of lysate protein with 2.5 μg of monoclonal antibody. Immunoprecipitates were fractionated by SDS-PAGE (10% gel), and PKC-α was visualized by Western blot analysis. C, Cultures were incubated for 1 hr with 1 μM okadaic acid before the addition of 1 μM Bryo as indicated. At 16 hr later, they were extracted with LB, and proteins (30 μg) were fractionated by SDS-PAGE (7% gel). PKC-ϵ was visualized by Western blot analysis. ▲ and △, 90- and 86-kDa PKC-ϵ bands, respectively.

the proteasome inhibitors spare multiubiquitinylated PKC-α from degradation.

Lacta preserved >90-kDa PKC-ϵ species produced by Bryo. Fig. 6B shows that Bryo induced the production of >90-kDa PKC-ϵ species, which accumulated in the presence of Lacta. Thus, it is likely that the >90-kDa species are ubiquitinylated because they accumulated in the presence of Lacta. We were unable to detect ubiquitinylated PKC-ϵ with the 1B3 and 2C5 antibodies, and the 4F3 antibody is no longer available. The cells seem to contain less than one fifth as much of the ϵ as of the α isoform, as estimated by Western blot analysis with purified recombinant PKC-α and PKC-ϵ as standards.[1] Apparently, an insufficient amount of multiubiquitinylated PKC-ϵ accumulated during the Bryo-plus-Lacta treatment to detect with the antibodies.

Discussion

The following observations support the hypothesis that the Ub/proteasome system is primarily responsible for the intracellular degradation of a novel and a conventional isoform of PKC and, by inference, several other isoforms. First, Western blot analysis indicated that Bryo produced PKC-α and -ϵ species in human fibroblasts that were larger than the native isozymes. Immunostaining with different Ub antibodies confirmed that the larger PKC-α species are multiubiquitinylated (Figs. 5 and 6). Previously, we showed that Bryo induced ubiquitinylation of PKC-α in both *in vivo* experiments with epithelial cells and *in vitro* (16). Second, two structurally diverse proteasome inhibitors, Lacta and ZGLALal, spared PKC-α and -ϵ from down-regulation by Bryo or PMA in human fibroblasts (Figs. 2 and 3). Lacta also spared autophosphorylated PKC-α, which is the active form of the kinase *in vivo* (Fig. 4A). Perhaps Ub carboxyl-terminal hydrolases regenerated active kinase from ubiquitinylated PKC. Alternatively, inhibition of the proteasome may deplete free Ub, which is recycled as ubiquitinylated proteins are degraded (17), and thereby prevent further ubiquitinylation. Third, Ub immunostaining showed that the combination of Bryo plus Lacta synergistically increased multiubiquitinylated ~180-kDa PKC-α (Fig. 6A). This is the result expected if Bryo induces ubiquitinylation of PKC and Lacta blocks its degradation. Finally, inhibitors of calpains, cathepsins, lysosomal proteolysis, and vesicle trafficking did not affect down-regulation of PKC-α or -ϵ (Fig. 1). Note that the Ub/proteasome pathway apparently makes a major contribution to down-regulation at lower and higher doses of Bryo and PMA. Lacta substantially spared total PKC activity and PKC-α protein from down-regulation by 50 nM Bryo or 100 nM PMA (Fig. 3)

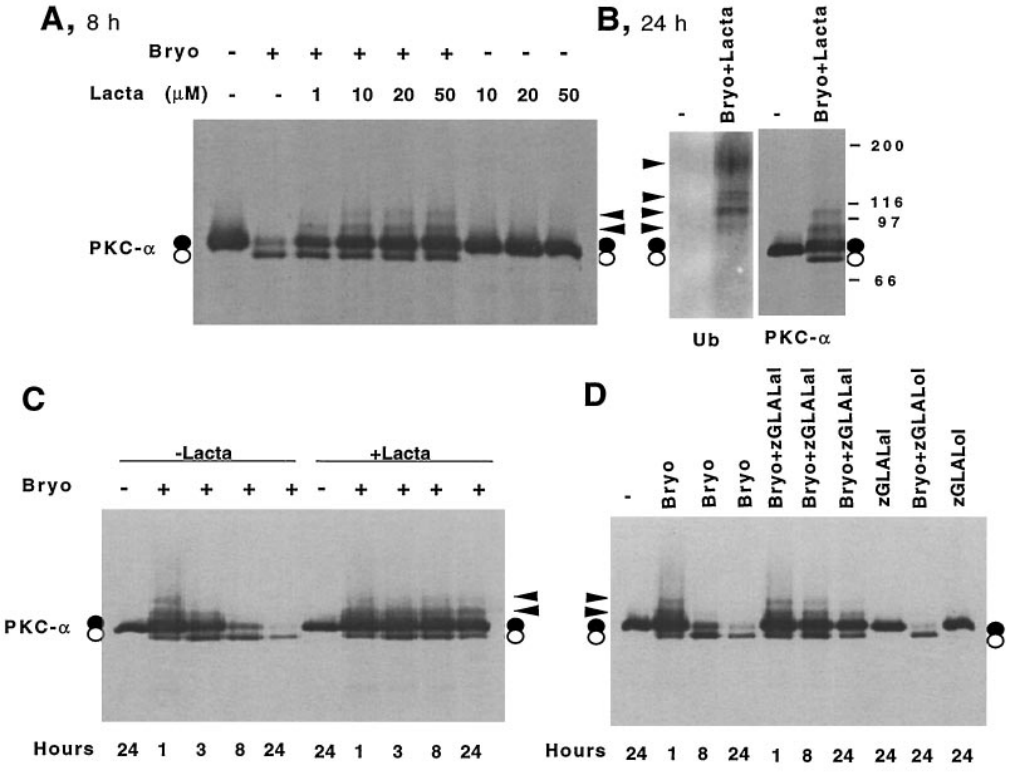

Fig. 5. Bryo produces >80-kDa ubiquitinylated PKC-α species that are preserved by proteasome inhibitors. A, Cultures were incubated with the indicated concentration of Lacta for 1 hr before the addition of 1 μM Bryo as indicated. At 8 hr later, PKC-α was extracted with LB and immunoprecipitated from 0.23 mg of protein with 2.5 μg of antibody. Immunoprecipitated proteins were separated by SDS-PAGE (10% gel), and PKC-α was visualized by Western blot analysis. B, Cultures were incubated in the presence or absence of 50 μM Lacta for 1 hr before the addition of 1 μM Bryo as indicated. At 12 hr later, the cultures were extracted with LB, and PKC-α was immunoprecipitated from 2 mg of lysate protein with 10 μg of antibody. Ubiquitinylated proteins were visualized by Western blot analysis with 4F3 antibody. PKC-α bands were immunostained after detection of ubiquitinylated proteins. C and D, Cultures were incubated with 50 μM Lacta (C) or a 50 μM concentration of the indicated peptidyl aldehyde or alcohol (D) for 1 hr before the addition of 1 μM Bryo. After the indicated interval, proteins were extracted with LB, and PKC-α was immunoprecipitated from 0.4 (C) or 0.2 (D) mg of lysate with 2.5 μg of antibody. Immunoprecipitated proteins were fractionated by SDS-PAGE (10% gel), and PKC-α was visualized by Western blot analysis. Molecular mass markers are indicated (kDa). ● and ○, 80- and 76-kDa PKC-α bands, respectively. *Arrowheads*, ubiquitinylated PKC-α bands.

and spared PKC-α and PKC-ε proteins from down-regulation by 1 μM Bryo or PMA (Fig. 2).

Some reports have implicated calpains in the down-regulation of PKC (22, 23). Most notably, Eto *et al*. (22) showed that AcLLNal (calpain inhibitor I) and a 27-mer calpastatin peptide inhibited the disappearance of PKC-ε produced by TRH in pituitary GH_4C_1 cells. However, AcLLNal is a moderately potent inhibitor of the 26S proteasome (28), and E64d, which is a cell-permeant cysteine protease inhibitor, did not affect TRH-induced down-regulation of PKC-ε (22). Curiously, a 34-mer calpastatin peptide (the 27-mer with seven additional carboxyl-terminal residues) inhibited the degradation of Mos, which is known to be multiubiquitinylated and degraded by the proteasome (35). Calpain either plays a role in PKC-ε and Mos degradation, which seems unlikely because of their insensitivity to calpain inhibitors other than the calpastatin peptides (22, 35, and current report), or treatment with the calpastatin peptides inhibited the Ub/proteasome pathway. Calpastatin peptides may independently block the Ub/proteasome pathway and calpains because calpastatin and Ub share some amino acid sequences (36). Neither E64d nor calpain inhibitor II (AcLLMal) affected the disappearance of PKC-α or -ε evoked by Bryo in human fibroblasts (Fig. 1). Interestingly, AcLLNal preserved PKC-α and -ε from down-regulation by Bryo (Fig. 1), which is in agreement with the preservation of PKC-ε from down-regulation by TRH (22). Although AcLLNal and AcLLMal are nearly equipotent calpain inhibitors, AcLLNal is much more potent toward proteasomal peptidase activities than AcLLMal (28). Studies of *m*-calpain-sensitive and -resistant mutants of PKC-α expressed in COS-1 cells suggest that *m*-calpain is not responsible for down-regulation produced by PMA (20). Taken together, these findings suggest that the down-regulation of PKC-α or -ε by the Ub/proteasome pathway is independent of calpains.

Polypeptide segments, dubbed PEST sequences, are known to target proteins for degradation by the proteasome (for a review, see Ref. 18). PEST sequences are hydrophilic segments that contain at least one proline, one glutamic acid or aspartic acid, and one serine or threonine. They lack positively charged residues (lysine, arginine, or histidine), which flank the sequence. Table 1 shows PEST sequences in conventional (α, β, and γ), novel (δ, ε, η, and θ), and atypical (ι and ζ) PKC isoforms. All of the isoforms except PKC-δ have one or more sequences with positive PEST-FIND scores (Table 1). Other peptide motifs, such as the cyclin destruction box, KFERQ motifs, and threonine-proline or serine-proline pairs, also can target proteins for destruction by the proteasome (18). Although it is not known how PEST sequences are recognized, protein kinases and phosphatases recognize

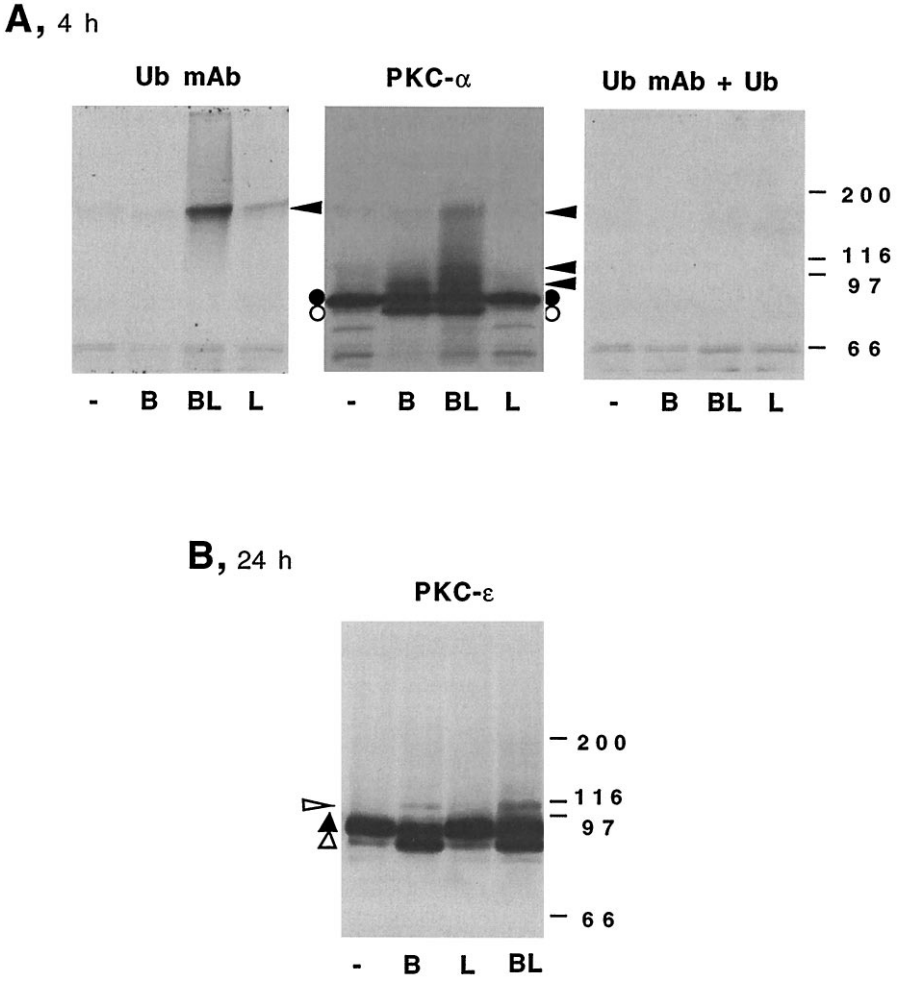

Fig. 6. Bryo and Lacta synergistically increase ubiquitinylated PKC-α and >90-kDa PKC-ϵ species. Cultures were incubated with 50 μM Lacta (*L*) for 1 hr before the addition of 1 μM Bryo (*B*) as indicated. At 4 hr (A) or 24 hr (B) later, proteins were extracted with LB containing 5 mM *N*-ethylmaleimide, and PKC-α was immunoprecipitated from 2 mg of lysate with 10 μg of PKC-α (A) or PKC-ϵ (B) antibody. Immunoprecipitates were fractionated by SDS-PAGE (7% gel) and transferred to nitrocellulose, and ubiquitinylated proteins were visualized with the combination of 1B3 and 2C5 antibodies (*Ub mAb*). The blots were stripped and immunostained for PKC-α or PKC-ϵ as indicated. The mouse immunoglobulin chains are absent because they ran off the gel, which was electrophoresed for 525 V-hr to resolve the faster and slower PKC-α and -ϵ bands. After immunostaining for PKC-α, the blot was stripped, autoclaved, and immunostained with the 1B3 and 2C5 antibodies in the presence of 0.2 mg/ml purified Ub (*Ub mAb + Ub*). Finally, the blot was stripped, autoclaved, and immunostained again with the 1B3 and 2C5 antibodies. The last immunostaining (not shown) primarily identified the 180-kDa band in the Bryo plus Lacta (*BL*) sample, similar to the first immunostaining. Molecular mass markers are indicated (kDa). ● and ○, 80- and 76-kDa PKC-α bands, respectively. ▲ and △, 90- and 86-kDa PKC-ϵ bands, respectively. *Arrowheads*, ubiquitinylated PKC-α species and >90-kDa PKC-ϵ band.

TABLE 1

PEST sequences of human PKC isoforms

The PEST-FIND algorithm (18) was used at the Genetic Data Environment WWW server at the Institute for Molecular Biotechnology (Jena, Germany). PEST sequences with scores of ≥1 are shown.

PKC isoform	PEST sequence	PEST score
α	91KGPDTDDPR99	13.8
β-I	1MADPAAGPPPSEGEESTVR19	8.9
β-II	91KGPASDDPR99	1.9
	276KLLSQEEGEYFNVPVPPEGSEANEELR302	2.4
γ	90KGPQTDDPR98	4.5
	316RMGPSSSPIPSPSPSPTDPK335	16.2
δ	None found	
ϵ	322KLIAGAESPQPASGSSPSEEDR343	8.2
η	649KEEPVLTPIDEGH661	2.9
θ	339REPQGISWESPLDEVDK355	4.7
ζ	51KWVDSEGDPCTVSSQMELEEAFR73	1.2
	86HVFPSTPEQPGLPCPGEDK104	3.6
	175HMDSVMPSQEPPVDDK190	4.1
	190KNEDADLPSEETDGIAYISSSR211	6.4
	521KQALPPFQPQITDDYGLDNFDTQFTSEPVQLTPDDEDAIK560	3.3
ι	60KWIDEEGDPCTVSSQLELEEAFR82	2.0
	531KPNISGEFGLDNFDSQFTNEPVQLTPDDDDIVR563	1.7

some PEST and non-PEST segments that target proteins for rapid degradation. Phosphorylation of certain threonine or serine residues triggers ubiquitinylation of several proteins whose degradation by the proteasome is inducible by external stimuli [e.g., IκB-α, cyclins, and the c-Fos/c-Jun heterodimer (17, 18, 37–39)]. Dephosphorylation of Ser3 of Mos evokes ubiquitinylation of Lys34 and degradation by the proteasome (35). Stimulus-induced dephosphorylation and degradation of PKC seems to be analogous to Mos.

Previously, we proposed that nonphosphorylated PKC-α is an intermediate in the down-regulation pathway in renal epithelial cells (14, 16). Bryo produced nonphosphorylated

76-kDa PKC-α and an analogous 86-kDa form of PKC-ε in human fibroblasts (Figs. 2A, 4, and 6). Ubiquitinylation of PKC-α *in vitro* required the nonphosphorylated 76-kDa form of the kinase (16). Okadaic acid, a selective inhibitor of phosphatases PP1 and PP2A (32), decreased Bryo-evoked production of 86-kDa PKC-ε and inhibited down-regulation (Fig. 4C). Although okadaic acid slightly affected down-regulation of PKC-α, the nonselective phosphatase inhibitor orthovanadate clearly decreased production of 76-kDa PKC-α and inhibited down-regulation (Fig. 4B). Okadaic acid selectively antagonized Bryo-induced down-regulation of PKC-ε, apparently by inhibiting its dephosphorylation. The selectivity of okadaic acid toward the ε isoform of PKC suggests that the involvement of different phosphatases, at least in part, accounts for the slower and less efficient down-regulation of PKC-ε relative to PKC-α. Okadaic acid induces ubiquitinylation and degradation of IκB-α *in vivo* (40) and only slightly affected the Bryo-induced down-regulation of PKC-α, which shows that the phosphatase inhibitor does not usually suppress the Ub/proteasome pathway. These findings support the view that dephosphorylation produces 86-kDa PKC-ε and that dephosphorylated forms of PKC-α and -ε are obligatory intermediates in down-regulation. The relationship between the phosphorylation state of certain threonine or serine residues of PKC and ubiquitinylation remains to be clarified.

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