Differential Regulation of Protein Kinase C Isozymes by Thyrotropin-Releasing Hormone in \( \text{GH}_4\text{Cl} \) Cells

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\( \text{GH}_4\text{Cl} \) cells, which express \( \text{Ca}^{2+} \)-dependent \( \alpha \)- and \( \beta \)- as well as \( \text{Ca}^{2+} \)-independent \( \gamma \), \( \epsilon \), and \( \zeta \) protein kinase C (PKC) isoforms, provide a cell culture model for studying isoform-specific properties and functions. Hormonal activation of PKCs regulates the differentiated functions of these cells, namely secretion and synthesis of prolactin (PRL). We previously reported that thyrotropin-releasing hormone (TRH) selectively down-modulates \( \epsilon \)-PKC with no effect on \( \alpha \) or \( \beta \)-PKCs (Kiley, S. C., Schaad, D., Parker, P., Hsieh, L.-L., and Jaken, S. (1990) J. Biol. Chem. 265, 15704-15712). We now extend these studies to explore the relationship between TRH-stimulated diacylglycerol (DAG) levels and \( \epsilon \)-PKC down-modulation. TRH stimulates three distinct DAG phases in \( \text{GH}_4\text{Cl} \) cells. Phase 1 DAG peaks at 15 s, is accompanied by a 6-fold increase in intracellular \( \text{Ca}^{2+} \), and causes the redistribution of \( \alpha \), \( \beta \), \( \gamma \), and \( \epsilon \)-PKC isoforms from a soluble to a detergent-insoluble particulate compartment. Phase 2 DAG peaks at 10 min, is not associated with a \( \text{Ca}^{2+} \) signal, and does not activate PKC by any criteria tested. Phase 3 DAG peaks at 6 h and is sustained through 12 h. This novel DAG formation is not associated with increased intracellular \( \text{Ca}^{2+} \). The time course of phase 3 DAG formation corresponds to the time course of TRH-stimulated \( \epsilon \)-PKC down-regulation; maximal effects are observed at 6-12 h for both events. Unlike \( \alpha \), \( \beta \), and \( \gamma \)-PKCs which are preferentially distributed in the soluble fraction of resting \( \text{GH}_4\text{Cl} \) cells, \( \epsilon \)-PKC is also distributed in the detergent-insoluble particulate fraction. The selective compartmentalization of \( \epsilon \)-PKC in the particulate fraction may render this pool uniquely susceptible to proteolytic degradation. The time course of phase 3 DAG formation and \( \epsilon \)-PKC down-modulation corresponds to the time course of decreasing PRL message synthesis in \( \text{GH}_4\text{Cl} \) cells. The data suggest that loss of \( \epsilon \)-PKC may be associated with the down-regulation of prolactin synthesis and that regulation of PRL gene transcription may be an \( \epsilon \)-PKC-specific function in \( \text{GH}_4\text{Cl} \) cells.

that protein kinase C (PKC)\(^1\) is directly involved in the regulation of prolactin synthesis and secretion in these cells. Tumor-promoting phorbol esters which bind to and activate PKC (2) stimulate these responses (3). Thyrotropin-releasing hormone (TRH), a physiological regulator of pituitary cell function, also stimulates prolactin secretion and synthesis in \( \text{GH}_4\text{Cl} \) cells (4, 5). TRH activates phospholipase C mediated phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) hydrolysis resulting in increased cellular levels of two PKC cofactors, diacylglycerol (DAG) and \( \text{Ca}^{2+} \) (6-9). The time course of TRH-mediated PKC redistribution from the soluble to the particulate subcellular fraction corresponds to the time course of PIP\(_2\) hydrolysis (10-13). Redistribution from the soluble to the particulate fraction is considered the first step in PKC activation (10-13). Hence, TRH receptor occupancy is coupled to PKC activation in \( \text{GH}_4\text{Cl} \) cells.

Protein kinase C is a growing family of enzymes which can be grouped into two categories based on \( \text{Ca}^{2+} \) requirements for activation: \( \text{Ca}^{2+} \)-dependent and \( \text{Ca}^{2+} \)-independent. PKC isoforms \( \alpha \), \( \beta \), and \( \gamma \) are \( \text{Ca}^{2+} \)-dependent and phospholipid-dependent kinases (14-16), whereas, the \( \delta \), \( \epsilon \), and \( \zeta \)-isozymes are \( \text{Ca}^{2+} \)-independent, phospholipid-stimulated kinases (17-20). Isozyme-specific properties such as substrate specificities (14, 19, 21-23), subcellular locations (24-28), activation requirements (14-16, 21-23, 29, 30), and rates of down-modulation (30-33) suggest that different isoforms may perform unique cellular functions. TRH-associated responses in \( \text{GH}_4\text{Cl} \) cells can be categorized as \( \text{Ca}^{2+} \)-sensitive or \( \text{Ca}^{2+} \)-insensitive (reviewed in Ref. 1). The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; PIP\(_2\), phosphatidylinositol 4,5-bisphosphate; IP\(_3\), inositol 1,4,5-trisphosphate; TRH, thyrotropin-releasing hormone; PDBu, phorbol dibutyrate; TEC, thin-layer chromatography; PMSF, phenylmethylsulfonfyl fluoride; PBS, phosphate-buffered saline; FBS, fetal bovine serum; EGTA, ethylenebis(oxyethylenenitrito)tetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; Hapes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; DMEM, Dulbecco's modified Eagle's medium; Pipes, 1,4-piperazinediethane-sulfonic acid; RIPA, radioimmuno precipitation buffer; mAb, monoclonal antibody.

\(^1\) Kiley, S. C., Parker, P. J., Fabbro, D., and Jaken, S. (in press).

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The second phase is sustained for 210 min (34-36) and is not associated with an appreciable elevation of cytosolic Ca\(^2+\) (37, 38). The lack of a Ca\(^2+\) signal associated with phase 2 DAG raises the possibility that phase 2 DAG could preferentially activate Ca\(^2+\)-independent PKCs. Redistribution of total PKC activity correlated with first, but not second phase DAG in GH cells (10-13, 36). The effects of the two DAG phases on individual PKC isozymes was not evaluated in those studies. In other studies, the time courses of redistribution for Ca\(^2+\)-independent \(\varepsilon\)PKC as well as Ca\(^2+\)-dependent \(\alpha\) and \(\delta\)-PKCs correspond to phase 1, but not phase 2 DAG (30). These data demonstrate that TRH, like phorbol ester, is not an isozyme-selective agonist. However, unlike phorbol esters, which down-modulate all GH cell PKC isozymes, prolonged TRH treatment selectively down-regulated \(\varepsilon\)-PKC, but not \(\alpha\)- or \(\delta\)-PKCs in GH cells (30, 39). Thus, a natural agonist such as TRH may in fact differentially regulate the activities of PKC isozymes during normal cellular responses.

The purpose of these studies was to explore the potential mechanism leading to \(\varepsilon\)-PKC selective down-modulation. Down-modulation by prolonged phorbol ester treatment has been shown to result from prolonged activation of \(\varepsilon\)-PKC (31, 40, 41). PKC is degraded more rapidly in stimulated cells than in resting cells (40-43). In the absence of effects on synthetic rates, the increased proteolysis eventually leads to depletion (i.e. down-modulation) of cellular PKCs. We have found that prolonged TRH-treatment produces a previously unreported third phase of DAG accumulation which temporally corresponds to \(\varepsilon\)-PKC down-modulation. Evidence for activation of \(\varepsilon\)-PKC by either redistribution or by phosphorylation could not be demonstrated. Down-modulation of \(\varepsilon\)-PKC was due to increased degradation in the presence of TRH. Down-modulation of \(\delta\)-PKC, another calcium-independent isozyme expressed in GH\(_C\) cells did not occur. Thus, the selective down-modulation of \(\varepsilon\)-PKC by TRH suggests that this isozyme plays a unique role in PKC signal transduction in GH cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following materials were obtained from the sources indicated: diolene and bovine heart cardiolipin, Avanti Polar Lipids (Birmingham, AL); octyl-\(\beta\)-d-glucopyranoside and ionomycin, Calbiochem (La Jolla, CA); sn-1,2-diacylglycerol kinase from Escherichia coli, Lipidex (Westfield, NJ); scored 20-20 cm silica Gel G TLC plates, Analtech (Newark, NJ); fura-2 AM, Molecular Probes (Eugene, OR); organic solvents, Burdick and Jackson (Muskegon, MI) and J. T. Baker (Phillipsburg, NJ); [\(^{32}\)P]ATP (5 Ci/\(\mu\)mol) and [\(^{32}\)P]P (5 Ci/\(\mu\)mol) in water (1 Ci/mmol), Du Pont-New England Nuclear (Boston, MA); and [\(^{32}\)S]methionine (1160 Ci/\(\mu\)mol), ICN (Irvine, CA). Phosphate-free Dulbecco’s modified Eagle’s medium, protein A-Sepharose, and chemicals, unless stated otherwise, were from Sigma. Ham’s F10 medium, methionine-free Dulbecco’s modified Eagle’s medium, horse serum, fetal bovine serum, and Immunoprecipitin (Formalin-fixed and J. T. Baker; 20-cm Silica Gel TLC plates, Analtech (Newark, NJ); thoridol conjugated mouse anti-rabbit IgG, goat anti-rabbit IgG, and color development kit were from Promega (Madison, WI). Biotinylated mouse anti-IgG and avidin-biotin alkaline phosphatase reagents were from Vector Laboratories (Burlingame, CA). Affinity-purified dichlorotriazinylamino-fluorescein-conjugated goat anti-mouse IgG and goat anti-rabbit IgG and affinity-purified mouse IgG and rabbit anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Airsol was from Air Products and Chemicals (Allentown, PA). Monoclonal antibody to \(\varepsilon\)PKC was from Seikagaku America (Rockville, MD). Polyclonal antiserum for antibody to the carboxylterminal peptide antiserum was obtained from Doriano Fabbro (Basel, Switzerland). Thapsigargin was purchased from LC Services (Woburn, MA).

**Cell Culture—**GH\(_C\) cells were grown on Ham’s F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (PBS) without antibiotics at 37°C in a 5% CO\(_2\)-air atmosphere. Unless stated otherwise, cells were cultured 5-7 days prior to use with medium changes every other day.

**Quantitation of Cellular DAG**—24 h prior to hormone treatment, GH cells were seeded 4-6 x 10\(^5\) cells/60-mm cell culture dish and medium was changed 8-10 h later. Thyrotropin-releasing hormone (300 nm) was added to cultures for time indicated. After treatment, cells were transferred to an ice tray and washed twice with ice-cold Dulbecco’s PBS (1.1 mM monobasic potassium phosphate, 8.1 mM dibasic sodium phosphate, 2.7 mM potassium chloride, 138 mM sodium chloride, 0.9 mM calcium chloride, and 0.5 mM magnesium chloride), followed by the addition of 0.5 ml of ice-cold methanol containing 0.5 mg of boric acid/ml (44) for 5 min at -20°C. Fixed cells were scraped from culture dishes along with an additional 0.6 ml of methanol/borrate wash and lipids were extracted according to the method of Bligh and Dyer (45). Chloroform layers were transferred to 1.5 ml of conical polypropylene tubes and dried under nitrogen. In several experiments, DAG was semipurified from crude lipids on silicic acid columns (46) using heptane instead of hexane (47).

DAG mass was quantitated using the DAG kinase method of Preiss et al. (48). [\(^{32}\)P]Phosphatic acid was detected using the short-bed/continuous development TLC method of Welsh and Schmeichel (44). [\(^{32}\)P]PA spots were scraped into scintillation vials, 8-ml Aquasol-2 scintillation fluid (Du Pont-New England Nuclear) was added to each vial, and samples were counted on a Beckman LS-1801 scintillation counter. The amount of cellular DAG was calculated from the specific activity of [\(^{32}\)P]ATP used per assay and the diolein standard curve.

**Preparation of Digitation-soluble and Insoluble Fractions**—Where indicated, 35-mm culture dishes of GH cells were treated with TRH or phorbol dibutyrate (PDBu) at 37°C. Coverslips were placed in a cuvette at a 45° angle to the excitation light path, washed three times in balanced salt solution (130 mM sodium chloride, 0.9 mM calcium chloride, and 0.5 mM magnesium chloride), 5.6 mM glucose, 10 mM Hepes, pH 7.4, and 1.5 mM calcium chloride (37) supplemented with 0.1% bovine serum albumin (BSA) and prewarmed to 37°C. Coverslips were transferred to the cuvette for 3-5 min. Subsequently, Fmin was determined by adding 50 mM potassium chloride (K\(^+\)) to the cuvette for 3-5 min. Subsequently, Fmax was determined by adding 7.5 mM Tris-EGTA and Tris base to pH 5.3 for 7 min. Spectrofluorometric analysis was performed using a Shimadzu Model RF-5000 U spectrofluorophotometer. [Ca\(^{2+}\)]\(_i\), was calculated from basal and agonist-stimulated fluorescence (F) as K\(_d\) x (F - F\(_{\text{min}}\)) / (F\(_{\text{max}}\) - F) with the K\(_d\) taken to be 224 nm (49).

**Preparation of Digitation-soluble and Insoluble Fractions**—Where indicated, 35-mm culture dishes of GH cells were treated with TRH or phorbol dibutyrate (PDBu) at 37°C. All subsequent steps were performed at 4°C. Coverslips were washed twice for 30 s with microtubule stabilization buffer [MSB; 0.1 M Pipes, pH 6.9, containing 1 mM EGTA, 1 mM magnesium acetate, and 2 mM glycerol with 3 mM ATP, 1.5 mM a-protein and 1.5 mM phenylmethylsulfonyl fluoride (48).**

It should be noted that cell culture conditions, particularly serum supplements, can markedly affect DAG mass values and even inhibit DAG responses. The data shown in Fig. 1 were generated from the lipid extracts of cells treated in the presence of serum. These DAG mass values are two to three times greater than the mass values determined for an equal number of cells grown in serum-free medium. In subsequent experiments, a new lot of horse serum caused the elevation of basal DAG levels. The disappearance of this elevation was restored by treatment with PDBu. All three DAG phases were restored and basal DAG levels reduced by growing cells in serum-free medium for 24-48 h prior to TRH treatment.
were treated with 32P/ml medium for 4 h and the supernatant reserved. The digitonin-insoluble fraction was centrifuged at 5,800 g for 20 min and the supernatant was discarded. Detergent extraction according to the method of Wessel and Fliigel (54).

**Preparation of Cell Lysates**—Where indicated, cultures (35-mm dishes) were treated with TRH at 37 °C and subsequent steps were performed at 4 °C. Lysates were prepared by sonicating in buffered sucrose as % w/v and described (51). The protein concentration of cell lysates were determined by the method of Bradford (52). Lysate protein was precipitated with 10% trichloroacetic acid for 10 min on ice and precipitates were collected by centrifugation at 3000 × g for 5 min, washed once with diethyl ether, recentlyrifuged and air-dried. Protein precipitates were solubilized in Laemmli buffer (51) and the pH was adjusted with 1 M Tris.

**Immuno blot Analysis**—Samples in Laemmli buffer were electrophoresed on 7.5% polyacrylamide gels and then electrophoretically transferred to nitrocellulose. Nitrocellulose blots were blocked on 5% skim milk and then incubated in 2 ml of the same medium containing 10% FBS. Cultures were washed three times in phosphate-free DMEM/O.2% FBS and incubated with 1% FBS for 60 min in a 37 °C CO2 incubator. Times and concentrations of [35S]methionine labeling and TRH treatment regimens were complete, cultures were washed twice in PBS. Cells were scraped into 0.5 ml of RIPA buffer (4 °C) containing 2 μl leupeptin, 1.5 μg aprotinin, 1 mM PMSF, 50 mM sodium fluoride, and 100 mM sodium vanadate. Culture dishes were washed with another 0.5 ml RIPA and the wash was combined with scraped cells. RIPA lysates were homogenized with 15 passes through a 26-gauge needle fitted to a 1-ml syringe.

**Immunoprecipitation of Cellar α- and ε-PKC**—Samples (1 ml) were precleared by incubating with 5% FBS and 310 μl of formalin-fixed Staph A cells (20% v/v in RIPA) for 60 min at 4 °C. Equal aliquots of the precleared supernatants were incubated with α- or ε-PKC-specific antibody preparations. For α-PKC, 3 μg of purified M6 mAb was used per assay and 3 μg of affinity-purified mouse IgG was used as a nonimmune control. ε-PKC was immunoprecipitated with 2 μg of affinity-purified rabbit anti-ε peptide antibody and affinity-purified rabbit anti-mouse IgG was used as the nonimmune rabbit IgG control. ε-Peptide antiserum was affinity-purified using peptide coupled to Affi-Gel 10 (Bio-Rad). Immune complexes were precipitated by incubating samples with 40 μl of a 50% protein A-Sepharose slurry for 60 min at 4 °C on a rocker platform. In the case of mouse antibody precipitations, protein A-Sepharose was first equilibrated with rabbit anti-mouse IgG. Immunoprecipitates were collected by centrifugation at 11,600 × g for 5 min. To reduce nonspecific background, precipitates were washed twice in RIPA, once in low salt buffer (50 mM Heps, pH 7.4, 150 mM sodium chloride, 0.1% Nonidet P-40, 1 mM EDTA, and 0.1% SDS), once in high salt buffer (50 mM Heps, pH 7.4, 500 mM sodium chloride, and 0.1% Nonidet P-40) and once more in low salt buffer. Immune complexes were eluted from protein A-Sepharose by boiling for 5 min in 35 μl of Laemmli buffer (51). Supernatants were collected and electrophoresed on 6% polyacrylamide gels. Gels were stained with Coomassie Blue and dried for autoradiography. Gels containing [35S]methionine-labeled samples were visualized in Amplify fluorography reagent (Amersham Corp.). Exposure of gels to film varied with isotope and experiment (see figure legends for details).

**RESULTS**

**TRH Treatment Stimulates a Triphasic DAG Response in GH4 Cells**—TRH has been shown to stimulate a biphasic DAG response in GH4 cells during the first hour of TRH treatment (34–36). To determine if down-modulation of ε-PKC, which occurs 4–6 h after TRH treatment of GH4 cells, correlated with DAG accumulation, TRH treatments and DAG mass measurements were studied over a longer time course. Prolonged TRH treatment produced a third phase of increased DAG mass (Fig. 1). Phase 1 DAG (15 ± 3) is associated with the rapid time course of phospholipase C-mediated PIP2 hydrolysis (6–9); phase 2 DAG (10 ± 3) phase 2 DAG (10 min) corresponds to the reported time course of phosphatidylincholine hydrolysis in GH4 cells (36); phase 3 DAG (6 ± 3) has not previously been reported in GH4 cells. Basal DAG levels changed very little over the 12-h time course. Under the conditions used, TRH did not influence cell growth. After 48 h of TRH treatment, cell numbers were 94 ± 10% (n = 2) of control cultures.

**Increased Intracellular Ca2+ Is Associated Only with Phase 1 DAG in Adherent GH Cells**—Previous studies have shown that TRH stimulates a rapid rise in intracellular calcium in GH cell suspensions (37, 38). Because the cytoskeletal organization of adherent and suspension cells is quite different, and because PKCs are associated with the cytoskeleton of GH cells (54), we measured the effect of TRH on intracellular Ca2+ in adherent GH cells.

In GH4 cells, TRH stimulated a rapid rise in intracellular Ca2+ from a basal value of 115–700 nM within 15 s after TRH treatment (Fig. 2A). Ca2+ levels decreased rapidly throughout the immunoprecipitation procedure.
Diacylglycerol Response Corresponds to ε-PKC Down-modulation

Redistribution of PKC from the soluble to the insoluble (particulate) subcellular compartment is considered the first step in PKC activation (10–13, 55, 56) and was used as a measure of PKC isozyme activation in response to TRH.

All four PKC isozymes were recovered predominantly in the digitonin-soluble fraction prepared from untreated cultures. With the exception of ε-PKC, very little immunoreactive PKC was found in the detergent-insoluble compartment of resting GH cells (Fig. 3). After a 15-s TRH treatment, there was a dramatic loss of PKCs from the soluble fraction with a concomitant increase in the insoluble fraction. Within 10 min of TRH treatment (which corresponds to the phase 2 DAG maximum), redistribution of PKC isoforms was no longer apparent. 6-h TRH treatment (which corresponds to the phase 3 DAG maximum) caused the selective loss (down-modulation) of ε-PKC, with no loss of immunoreactive α-, β-, or δ-PKCs.

Immunocytodiscsence studies shown in Fig. 4 verify the transient association of α- and ε-PKC with detergent-insoluble preparations after TRH treatment. A small amount of ε-PKC, but not α-PKC, was detectable in detergent-insoluble preparations of resting cultures. After a 15-s TRH treatment, a dramatic increase in α- and ε-PKC staining was observed at the cell peripheries. The intensity of fluorescence-staining gradually diminished to control levels within 15 min. Both immunofluorescence and immunoblot analyses indicated that the level of ε-PKC in the detergent-insoluble fraction of resting cells is greater than the levels of α-, β-, and δ-PKCs.

Phosphorylation of α- and ε-PKCs is Associated with Activation and Redistribution to the Detergent-insoluble Subcellular Compartment—In some cases, increased phosphorylation of α- and ε-PKCs is observed in response to TRH treatment. The reduced electrophoretic mobility of the δ-PKC doublet occurs in response to TRH treatment. The decreased electrophoretic mobility of ε-PKC seen in insoluble preparations appears to be due to transient phosphorylation of ε-PKC following 15-s TRH treatment (see Fig. 5).
ion of PKC has been shown to correlate with increased PKC activation (57, 58). Because increased DAG levels after a 10-min TRH treatment did not cause PKC redistribution, we used PKC phosphorylation state as an alternative assay for activation. α- and ε-PKCs were immunoprecipitated from 32P04-labeled resting or activated GH cell cultures (Fig. 5). α- or ε-PKCs could not be detected in the soluble fraction from either control or stimulated cultures, although substantial amounts of immunoreactive α- or ε-PKCs are present in these fractions (see Fig. 3). Immunoblots of precipitated samples indicated comparable amounts of immunoreactive α-PKC in control (untreated) soluble fractions and PDBu-treated insoluble fractions (data not shown). Phosphorylated α- and ε-PKCs were detected in the insoluble fractions after 15 s of TRH treatment, and diminished after 10 min of TRH treatment. These results demonstrate that an increase in PKC phosphorylation state occurs during phase 1 but not during phase 2 DAG.

Phosphorylated PKCs were also detected in the insoluble fractions of cultures stimulated with PDBu for 10 min. Thus, with both TRH and PDBu, phosphorylation correlated with redistribution. Both criteria for activation (i.e. phosphorylation and redistribution) indicate that the first, but not the second phase of TRH-stimulated DAG is an important regulator of cellular PKC activity.

**Time Course of TRH-stimulated ε-PKC Down-modulation**

Corresponds with the Time Course of Sustained Phase 3 DAG—TRH treatment time course studies and immunoblot analysis demonstrate that the loss of immunoreactive ε-PKC is apparent 3 h post-TRH treatment and is complete within 6–12 h (Fig. 6). This time course corresponds with that of TRH-stimulated phase 3 DAG. α-PKC levels, which are not significantly affected by prolonged TRH treatment, are shown for comparison. In a few experiments, TRH did slightly decrease α-PKC levels by 20–30% (see Fig. 3). However, this effect was always smaller than the effect on ε-PKC.

Prolonged TRH Treatment Does Not Affect the Rate of ε-PKC Synthesis—Another possible mechanism of PKC down-modulation would be an agonist-stimulated decrease in the rate of PKC synthesis. To test this hypothesis, GH cells were pretreated with TRH, then pulsed briefly with [35S]methionine to determine if TRH influenced the rate of ε-PKC protein synthesis. The results shown in Fig. 7 indicate that synthetic rates of α- and ε-PKCs are relatively constant regardless of TRH treatment.

**Prolonged TRH Treatment Increases the Rate of ε-PKC Proteolysis**—PKC down-modulation has been shown to correlate with agonist-induced increased rate of PKC proteolysis (40–43). [35S]Methionine pulse-chase studies were undertaken to determine if increased proteolysis was the mechanism of TRH-stimulated ε-PKC down-modulation. The results shown in Fig. 8 indicate that TRH increases the degradation rate of ε-PKC. The effect on ε-PKC is much larger than the effect on α-PKC. After a 6-h chase, 61% of the radiolabeled ε-PKC was recovered, and this was decreased to 39% by TRH. After a 12 h chase, 59% of the radiolabeled ε-PKC was recovered, and this was decreased to 16% by TRH. In contrast, after a 12-h chase, 59% of the radiolabeled α-PKC was recovered, and this was only slightly decreased to 41% by TRH.

**Diacylglycerol Response Corresponds to ε-PKC Down-modulation**

Whole cell lysates were prepared from cultures treated with 300 nM TRH for the times indicated. Lysates (100 μg/lane) were electrophoresed on 7.5% polyacrylamide gels then electrophoretically transferred to nitrocellulose. Immunoblots were probed with α- and ε-PKC-specific antibodies as described under "Experimental Procedures." Blots shown are representative of four independent experiments. Positions of molecular weight standards are indicated on the left.
the end of the treatment period, cultures were pulsed with 0.5 mCi of 

During the last hour of treatment, medium was replaced with methi-
cultures were treated with 300 nM TRH for the times indicated.

were electrophoresed on

showed precipitation with nonimmune IgG and

300 nM TRH. At the end of chase, RIPA lysates were prepared and

Cultures were labeled with 0.1 mCi of [3sS]methionine for 18 h.

molecular weight standards are shown on the

DISCUSSION
We have determined that TRH stimulates a triphasic DAG response in GH, cells. The first phase is associated with increased phosphoinositide hydrolysis and redistribution of Ca2+-independent δ- and ε-PKCs as well as Ca2+-dependent α- and β-PKCs from the detergent-soluble to the detergent-insoluble compartment. An increase in the phosphorylation state of α- and ε-PKCs was associated with redistribution and activation. The second DAG phase was not associated with a Ca2+ signal and did not cause redistribution or phosphorylation of any PKC isozyme. Hence, phase 2 DAG was nonactivating by any criteria tested. The third DAG phase also was not associated with a Ca2+ signal and correlated with selective down-modulation of ε-PKC. Our data indicate that two of the three TRH-stimulated DAG phases may regulate PKC activities: Phase 1 as a "generalized" pan-PKC activator of four GH cell isozymes, and Phase 3 DAG as an isozyme-specific down-modulator of ε-PKC.

Ca2+-independent as well as Ca2+-dependent PKC isozymes were activated during Phase 1 DAG, suggesting that the DAG species along with the Ca2+ signal produced during this time act as general PKC activators. Prolactin secretion, which involves a massive reorganization of the cytoskeleton for secretory granule release (60), is initiated during this time. Effects of TRH on the nucleus are also observed during the first minute of TRH treatment. PKC-dependent phosphorylation of a 23-kDa nuclear protein and an increase in nuclear prolactin mRNA synthesis begin during the first minute of TRH treatment and continue for 30 min (61, 62). Activation of all the PKC isozymes may be necessary to orchestrate so many cellular events that occur during the first few minutes of TRH treatment.

Phase 2 DAG has been correlated with TRH-stimulated PC hydrolysis in GH3 cells (36). Because there is no accompanying increase in cytosolic Ca2+ levels, we considered that this phase could potentially serve as a selective pathway for activating Ca2+-independent PKCs. However, we found no evidence for PKC activation during phase 2 DAG according to any criteria. Immunofluorescence and immunoblot analyses indicated that redistribution of all isozymes was no longer apparent after 10 min of TRH treatment. Furthermore, phosphorylation of α- and ε-PKCs was not observed. Our results are consistent with the results of Martin et al. (36) using GH3 cells and Leach et al. (63) using IIC9 fibroblasts. Phase 2

FIG. 7. TRH effect on the rate of ε-PKC synthesis. GH cell cultures were treated with 300 nM TRH for the times indicated. During the last hour of treatment, medium was replaced with methionine-free DMEM in the absence or presence of TRH as appropriate (0.33- and 1-h TRH treatments were in methionine-free DMEM). At the end of the treatment period, cultures were pulsed with 0.5 mCi of [3sS]methionine for 10 min, RIPA lysates were prepared for immunoprecipitation as described under "Experimental Procedures." Immunoprecipitates were subjected to 6% SDS-polyacrylamide gel electrophoresis and dried gels were exposed to x-ray film for 30 h without an intensifying screen. Lane 1 shows precipitation with nonimmune antibodies, and lanes 2-8 contain 3sS-labeled proteins immunoprecipitated with α- (top) or ε- (bottom) PKC antibodies. Positions of molecular weight standards are shown on the left.

FIG. 8. Effect of TRH on the rate of ε-PKC degradation. Cultures were labeled with 0.1 mCi of [3sS]methionine for 18 h. Cultures were then switched to nonradioactive methionine-containing medium for the (chase) times indicated in the absence or presence of 300 nM TRH. At the end of chase, RIPA lysates were prepared and samples were immunoprecipitated with α- or ε-PKC antibodies as described under "Experimental Procedures." Immunoprecipitates were electrophoresed on 6% polyacrylamide gels and dried gels were exposed to x-ray film for 16 h without an intensifying screen. Lane 1 shows precipitation with nonimmune IgG and lanes 2-8 contain 3S-labeled proteins immunoprecipitated with α- (top) or ε- (bottom) PKC antibodies. Positions of molecular weight standards are indicated on the left.

Addition of Ca2+ Agonists to Phase 3 DAG Does Not Result in Down-modulation of Ca2+-dependent PKC Isozymes—The selective effect of Phase 3 DAG on ε-PKC could be due to generation of a unique DAG species or to the absence of any criteria. Immunofluorescence and immunoblot analyses indicated that redistribution of all isozymes was no longer apparent after 10 min of TRH treatment. Furthermore, phosphorylation of α- and ε-PKCs was not observed. Our results are consistent with the results of Martin et al. (36) using GH3 cells and Leach et al. (63) using IIC9 fibroblasts. Phase 2
DAG does not activate PKC according to any activation parameter assessed.

Prolonged effects of TRH on GH cell DAG levels were carried out to determine if increased DAG could account for the TRH-mediated down-modulation of ε-PKC. Previous studies of TRH-stimulated DAG accumulation focused on effects during the first hour (34–36). Our results demonstrate a third phase of DAG accumulation between 4 and 6 h of TRH treatment. TRH does not increase growth of GH cell (5). Therefore, the increased DAG is not simply due to an increased lipid synthesis necessary for cell division. Phase 3 DAG temporally corresponds to down-modulation of ε-PKC. Both begin 3–4 h after TRH treatment, are maximal by 6 h, and are maintained for at least 12 h (compare Figs. 1 and 6). Coincidentally, this time course also correlates with that for attenuation of the TRH-mediated increase in nuclear prolactin mRNA (62). Further work is required to determine if down modulation of ε-PKC can account for the attenuated TRH effects on prolactin message synthesis.

A direct association between phase 3 DAG and ε-PKC down-modulation has not yet been established. There are now several reported examples in which down-modulation has been shown to be a consequence of prolonged PKC activation (40–49). In vitro studies have shown that phospholipids increase the susceptibility of PKCs to proteases (32, 64). Thus, down modulation is thought to be due to increased proteolysis of membrane-associated (activated) PKCs. According to this viewpoint, down-modulation can be considered an indirect measurement of PKC activation. This may explain the decreased PKC content of some transformed cells relative to their normal counterparts (65). Our results with ε-PKC do not fit this model. TRH did cause increased proteolysis of ε-PKC, however, only a rapid and transient redistribution could be measured.

Redistribution may not be an adequate measure of PKC activation in all cases. Increased phosphorylation of PKCs has also been associated with activation (57, 58, 66, 67); however, TRH caused only a rapid and transient increase in ε-PKC phosphorylation. Again, this could not be correlated with the time course of ε-PKC down-modulation. There are now several reports describing “translation-independent” PKC activation (68–71). These results imply there is a pool of particulate-associated, yet inactive PKC in resting cells. Under certain conditions which are not associated with an apparent redistribution from cytosol to particulate fractions, this pre-existing particulate form of PKC may become activated. It may be relevant that in GH3 cells, only the ε-PKC isoform is recovered in the detergent-insoluble fractions in appreciable amounts from resting cells. Compartmentalization of ε-PKC to the particulate fraction may, therefore, make it uniquely susceptible to the effects of TRH and phase 3 DAG. Continuous down-modulation of ε in the cytoskeleton would most likely lead to recruitment of soluble ε-PKC to the cytoskeleton. These combined events would explain the TRH-induced loss of ε-PKC from both subcellular compartments. Alternatively, phase 3 DAG may include a unique DAG species which selectively interacts with and modulates ε-PKC. In support of this possibility, dipalmitoylglycerol selectively increased the V_{max} of ε-PKC but not γ-PKC by 2-fold (23).

In addition to its differential regulation by TRH, ε-PKC differs from other GH cell PKCs with respect to substrate specificity, increased particulate association in resting cells, and apparent lack of involvement in agonist-stimulated reorganization of the actin cytoskeleton. These results are consistent with the hypothesis that different PKC isoforms perform different functions in GH cells. Our data indicate that ε-PKC has a unique role among GH cell isoforms with respect to signal transduction and cellular responses. Future studies will focus on the identification of molecular species found in phase 3 DAG as potential ε-PKC-specific agonists and the identification of ε-PKC-specific substrates. Such studies will be necessary to critically evaluate the role of ε-PKC in TRH-directed responses including regulation of PRL secretion and synthesis.

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REFERENCES
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