The Sustained Second Phase of Hormone-stimulated Diacylglycerol Accumulation Does Not Activate Protein Kinase C in GH3 Cells*

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Numerous hormones activate cells through receptor-regulated hydrolysis of phosphoinositides resulting in elevated cellular diacylglycerol (DAG), an activator of protein kinase C (PKC). Our previous studies showed that thyrotropin-releasing hormone (TRH) treatment of GH3 cells stimulated a rapid (10 s) but transient (<60 s) association of cytosolic PKC with the membrane. In this study, we investigated the roles of hormone-stimulated Ca++ and DAG levels in initiating and terminating the membrane association of PKC. The initial effects of TRH were not mimicked by elevating Ca++ levels, however, inhibiting TRH-stimulated Ca++ increases blocked hormone-stimulated PKC translocation. Hence, the TRH stimulation of both Ca++ and DAG levels were essential for the initial PKC translocation.

The termination of PKC membrane association could not be attributed to proteolysis of PKC nor to limiting Ca++ levels. Treatment of cells with phorbol diesters potentiated and prolonged the effects of TRH on PKC translocation, suggesting that DAG levels limited the membrane association of PKC. Since TRH stimulated a sustained increase in DAG levels, DAG composition was analyzed. There was a marked shift in DAG from tetraenoic to more saturated DAGs at longer times. In addition, increases in plasma membrane DAG in response to TRH were transient rather than sustained. We propose that the TRH stimulation of PKC translocation is short-lived due to the metabolism of plasma membrane DAGs which are effective in promoting PKC activation. In contrast, DAGs which accumulate in intracellular membranes during the sustained phase of TRH treatment appear to be ineffective as activators of PKC.

Cellular activation by numerous hormones is mediated through receptor-regulated hydrolysis of polyphosphoinositides leading to the intracellular elevation of calcium and diacylglycerol (DAG) (1). DAG generation is believed to activate protein kinase C (PKC), rendering the enzyme active at intracellular Ca++ concentrations (2).

Soluble PKC is known to bind to membranes, liposomes, or micelles in vitro under conditions of elevated Ca++ or in the presence of DAGs or phorbol diesters (3-5). Reversible Ca++-dependent membrane binding of PKC may occur in the absence of enzyme activation. In contrast, a stable chelator-insensitive, detergent-extractable membrane state is associated with enzyme activation (3-5). Treatment of intact cells with phorbol diesters, synthetic DAGs, or hormones results in the association of PKC with membranes in a chelator-insensitive, detergent-extractable form (6). This induced cytosol to membrane translocation may reflect in situ activation of PKC.

We previously reported that TRH treatment of GH3 pituitary cells results in the rapid but transient redistribution of PKC from a cytosolic to a membrane-associated form (7). Similar results were reported by Fearon and Tashjian (8). The dynamics of hormone-stimulated PKC translocation are poorly understood. TRH is known to rapidly trigger DAG accumulation (9, 10) and a cytoplasmic Ca++ rise (11, 12), but the precise role of these messengers in PKC activation has not been defined. The basis for the transient membrane association of the enzyme in TRH-stimulated cells is unknown. In other systems, it has been suggested that a transient membrane association may result from transient DAG generation (2), membrane-associated proteolytic cleavage of PKC (13), or release of the enzyme from the membrane upon PKC autophosphorylation or phosphorylation of membrane proteins (5, 14, 15).

In this study, we examined the relative roles of DAG and Ca++ in the TRH-triggered association of PKC with membranes. In particular, we sought to elucidate the basis for the transient nature of the membrane association of PKC in TRH-treated cells. Our results indicate that TRH-stimulated DAG production is sustained well beyond the period of PKC translocation but that there is a temporal switch from activating to nonactivating DAGs during TRH stimulation. We propose that this switch corresponds to the selective, compartmentalized metabolism of plasma membrane-associated, polyphosphoinositide-derived DAGs and the secondary generation of nonphosphoinositide-derived DAGs at intracellular membranes. The DAGs generated during the latter sustained phase of TRH action appear to be ineffective in stabilizing the membrane association of PKC.

**EXPERIMENTAL PROCEDURES**

*Materials*

DEAE-cellulose (DE52) was purchased from Whatman, Sephadex G-25 and G-75 resins from Pharmacia LKB Biotechnology Inc., and Ultrigel AcA 44 from IBF Biotechniques (Villeneuve-la-Garenne, France). Reagents for polyacrylamide gel electrophoresis and nitrocellulose for protein transfers was from Bio-Rad. 125I (carrier free) for antibody iodination was purchased from Amersham Corp. and Iodo-
Gen and Iodo-Beads from Pierce Chemical Co. Escherichia coli d-glyceride kinase was obtained from Lipidex, Inc. (Madison, WI). Fura-2/AM and Quin-2/AM were purchased from Molecular Probes, Inc. (Eugene, OR). All other reagents were purchased from Sigma.

**Methods**

*Production of Chicken Antibody to PKC—PKC was purified from rat brain cytosol by DEAR-cellulose chromatography and precipitated with potassium sulfate. Following desalting on Sephadex G-25, the material was loaded onto a phosphotyrosyl-serine-acylamidyl affinity column after rapidly adjusting the buffer to 1 mM Ca++. PKC was eluted from the resin as described (16). To remove 20-kDa contaminants, the enzyme was purified by gel filtration using an AcO-44 Ultragel column. The resulting enzyme preparation was 75% pure consisting of major 80-kDa bands and several minor 50-60-kDa bands (Ultragel fraction).

Young female white leghorn California grey hybrid chickens received 100 µg Ultragel fraction emulsified in incomplete Freund's adjuvant in weekly injections. Within 8 weeks, specific PKC antibody production was detected, and eggs collected during the fourth through sixth weeks were used for antibody preparation. The IgY fraction from yolks was prepared using a polyethylene glycol partitioning method described by Polson et al. (17).

*Purification and Characterization of Chicken Antibodies—*The immune IgY reacted predominantly with an 80-kDa band upon analysis of rat brain cytosol fractions by Western blotting techniques, however, several minor 50-60-kDa immunoreactive bands were also present. By affinity purification of antibodies from nitrocellulose blots of sodium dodecyl sulfate gels (18), the 50-60-kDa proteins were shown to be immunologically distinct from 80-kDa PKC. For the experiments of this study, monospecific anti-80-kDa antibodies were prepared by affinity purification of IgY using nitrocellulose blots of the 80-kDa protein (18). Proteolytic cleavage of purified PKC with trypsin indicated that the antibody reacted with several cleavage fragments of 62, 35, 24, and 17.5-20 kDa. By comparison with published studies (19), we infer that our antibody recognizes the amino-terminal regulatory domain rather than the carboxyl-terminal catalytic domain of PKC. Similar proteolytic fragments were detected when rat brain cytosol was autoclaved with Ca++. There were no immunoreactive fragments in the region of the gel corresponding to kinase M even though by enzyme assay maximal conversion to kinase M had occurred. The monospecific antibody reacted equally well with each of the three separated rabbit brain PKC isoenzymes (kindly provided by Dr. S. Jaken, Lake Placid, NY (19)).

*Cell Culture, Subcellular Fractionation, and Immunoblotting—*Monolayers of GH₃ cells; grown on 10-cm culture dishes, and experiments were conducted with 8 × 10⁶ cells/dish. Cultures were terminated by rapid aspiration and washing twice with ice-cold homogenization buffer. Cells were scraped into 0.25 ml of homogenization buffer (0.05 M Tris, pH 7.5, 0.25 M sucrose, 0.002 M EDTA, 10 µg/ml leupeptin) followed with a 0.25-ml wash of the dish. The cells were frozen-thawed in three cycles and centrifuged at 100,000 g for 60 min. Supernatants were removed as cytosol; pellet fractions were washed once with homogenization buffer, resuspended in the same and extracted with addition of Nonidet P-40 to 1%, incubated on 0 °C overnight and centrifugation at 100,000 × g for 60 min. Minor amounts of PKC were detected in the residual detergent-insoluble material.

Fractons were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% polyacrylamide gels and transferred to nitrocellulose using a Hoefer (San Francisco, CA) Transphor apparatus. Nitrocellulose sheets were incubated with 5% non-fat dry milk powder plus 0.05% Tween 20 in phosphate-buffered saline (0.04 M sodium phosphate, 0.15 M NaCl, pH 7.2) (Blotto) (21) for 1 h at room temperature and incubated overnight at 4°C with monospecific chicken anti-PKC antibody in Blotto. Sheets were washed four times with phosphate-buffered saline, 0.05% Tween 20 and incubated with second antibody for 4 h at room temperature either with [125I]-rabbit antichicken IgG or rabbit antichicken IgG-alkaline phosphatase dilituted in Blotto. Sheets were washed four times in PBS, 0.65% Tween 20 and visualized by autoradiography (Kodak X-AR film, Dupont Cronex lightening plus screen, overnight) for [125I] antibody or with 5-bromo-4-chloro-3-indolyl phosphate plus nitro blue tetrazolium for phosphatase-linked antibody (22).

Iodination of rabbit antichicken IgG was performed as follows. Fifty µg of Iodo-Gen in 50 µl of methylene chloride was coated onto the sides of a glass tube and four Iodo-Beads were added. One ml of IgG at 2 mg/ml in phosphate-buffered saline was introduced followed by 2 ml Na[125I]I. Following reaction for 5 min at room temperature, [125I]-IgG was recovered by gel filtration on Sephadex G-75.

Analysis of brain cytosol with phosphatase-conjugated second antibody is shown in Fig. 1A. An 80-kDa band was evident with monospecific antichicken antibody (lanes 1-3) whereas additional bands were evident with unpurified IgY (lane 2). Preimmune chicken IgY exhibited only nonspecific binding of several bands (Fig. 1A, lane 1). Analysis of GH₃ cell fractions with monospecific antibody and [125I]-second antibody is shown in Fig. 1B. In cytosol fractions (lanes 1-3), an 80-kDa band was detected. In membrane fractions (lanes 4-6), bands in addition to the 80-kDa band were detected; these were nonspecifically labeled since they were visualized when first antibody was omitted. The assay readily detected 5 ng of purified PKC; GH₃ cell cytosol was estimated to contain 0.4 µg of PKC/mg of cell protein. In each experiment, 80 µg of cytosol or 80 µg of membrane protein were loaded for gel electrophoresis. This represented 30% of each fraction prepared from each culture dish. All experiments except for that of Fig. 1A were conducted with [125I]-IgG as second reagent.

Quantitation was by densitometry with a Zeinher laser scanning densitometer. Data represent peak height determinations of 80-kDa bands. The data from single determinations are presented, however, these were representative of at least three independent experiments; replicates from separate culture dishes analyzed within a single experiment showed agreement within 20% of the signal.

*Determination of Celluar DAg Levels—*Total lipid extracts from cell monolayers grown on 6-cm dishes or from membrane fractions (see below) were analyzed for DAG using an E. coli DAG kinase assay (23). With diolein as standard, the assay was linear from 0.5-10 nmol. Plasma membranes were prepared and separated from intracellular membranes by Percoll gradient centrifugation as previously described (24). For analysis of DAG classes, [3H]Ptd from the DAG kinase assay was purified by thin layer chromatography, derivatized to dimetyl Ptd with diazenzoic acid and subjected to chromatography on AgNO₃-impregnated thin layer chromatography silica plates (24) with two ascending solvent systems (chloroform/methanol/water 60:30:3) followed by chloroform/methanol (9:1, v/v). Bands corresponding to polyunsaturated, tetraenoic, trienoic, dienoic, monoenoic, and saturated DAGs were scraped from the plate and quantitated by liquid scintillation counting.

*Determination of Cellular Calcium Levels—*Spectrofluorometric analyses of calcium levels with Quin-2 and Fura-2 were conducted by published methods (12, 25) using a Perkin-Elmer LS 5B fluorometer. IBM PC software for Fura-2 analysis was provided by Dr. Dean Brown, Perkin-Elmer Corp.

*Experimental Procedures.* Nitrocellulose sheets were incubated with monospecific chicken antibody (lane 1), immune IgY (lane 2), or monospecific anti-80-kDa IgY (lane 3). Rabbit antichicken IgG-alkaline phosphatase conjugate was used to visualize blots. B, GH₃ cell fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose. Monospecific anti-80-kDa antibody and rabbit antichicken IgG were used to identify PKC. Cytosol (15 µg, lanes 1-3) and membrane (90 µg, lanes 4-6) fractions were analyzed for control cells (lanes 1 and 4), cells treated with 1 µM TRH for 10 s (lanes 2 and 5), and cells treated with 16 µM PMA for 15 min (lanes 3 and 6).

**RESULTS**

Prior studies of hormone-stimulated PKC translocation have quantitated enzyme distribution by activity assay. Prob-
blems inherent in quantitation by enzyme assay include the presence of inhibitors (26), the necessity of chromatography with attendant recovery problems (6), the possibility of altered substrate specificity upon or following membrane association (27), and altered enzyme activity resulting from autophosphorylation (14) or proteolysis (19). In many translocation studies, total enzyme recovery for cytosolic and particulate fractions was less than 100%. We have utilized a highly specific immunochemical assay for PKC quantitation in unpurified cellular fractions which detects α, β, and γ PKC types.

**Effect of Tumor Promoters on PKC Distribution in GH3 Cells**—To further validate the immunoblot assay, the distribution of PKC was monitored following treatment of GH3 cells with phorbol diesters. As shown in Fig. 2A, PMA and phorbol 12,13-dibutyrate promoted a stable membrane association of PKC whereas the inactive phorbol diester 4α-phorbol 12,13-didecanoate did not. The relative potency of these agents compared favorably with previously reported studies (28).

PKC redistribution with maximally effective PMA concentrations was complete within 15 min of treatment (Fig. 2B). Approximately 80% of the cytosolic enzyme was transferred to the membrane fraction resulting in 85% of the total enzyme content in the membrane fraction. In subsequent studies, PMA treatment of parallel cultures was included in order to calibrate the effects of other agents (see legend to Fig. 3).

**Thyrotropin-releasing Hormone Induces a Transient Cytosol to Membrane Redistribution of PKC**—TRH treatment of GH3 cells led to a rapid transient redistribution of PKC (Fig. 3). TRH stimulated an increase in membrane PKC levels detectable within 5 s and maximal at 10 s. Following this initial increase, membrane levels decreased back to control by about 60 s (Fig. 3A and B). The ability of TRH to promote the membrane association of PKC was maximal by $10^{-6}$ M peptide and half maximal at about $4 \times 10^{-7}$ M (Fig. 3A). These results with an immunoblot assay confirm our previous studies which quantitated PKC levels enzymatically (7).

TRH treatment promoted a rapid transient decline in cytosolic PKC levels which was the reciprocal of changes in membrane levels (Fig. 3B). TRH stimulation for 10 s routinely stimulated the membrane stabilization of 20–40% of the cytosolic PKC, and there was a strict conservation of total cellular PKC at all time points. There were no detectable low molecular weight immunoreactive forms of PKC, and protease inhibitors (leupeptin and E 64) did not influence the redistribution of PKC. Therefore, it appeared that the cytosol to membrane redistribution promoted by TRH was transient with the reappearance of all of the molecules undergoing membrane translocation back into the cytosol. These results exclude the possibility that proteolysis terminates the membrane association of PKC.

The possibility that TRH was inactivated was excluded by the experiment shown in Fig. 3C where readdition of TRH (arrow) failed to promote an increase in membrane PKC levels. Restimulation with TRH following a washout promoted only a small transient rise in PKC levels (Fig. 3C).

**TRH Promotes a Sustained Increase in Cellular DAG Levels**—Since hormone-promoted DAG may be the principal trigger for PKC translocation to the membrane, the transient PKC response to TRH could have resulted from a transient generation of DAG. As shown in Fig. 4, the elevation of DAG with TRH was multiphasic with an initial rapid increase for 15 s followed by a plateau at about 60 s followed by a sustained increase for at least 10 min. Since DAG accumulation promoted by TRH was sustained rather than transient, changes in total cellular levels of DAG per se did not appear to account for the transient PKC redistribution. The possibility that mass determinations of DAG do not adequately assess molecular species or compartmentalized pools of DAG responsible for activating PKC will be addressed below.

**TRH Stimulation of PKC Redistribution Requires Hormone-induced Ca**2+ **Mobilization**—An additional possible basis for the rapid but transient membrane association of PKC stimulated by TRH involves the role of Ca**2+ and the ability of the hormone to promote a biphasic elevation of cytoplasmic Ca**2+ levels. As shown in Fig. 5A, TRH rapidly elevated Ca**2+ levels from 100 nM to a peak of 700 nM within 15 s. At beyond 60 s of TRH addition, Ca**2+ levels remained elevated at a plateau of 390 nM. The similarity in time courses for TRH-stimulated PKC translocation (Fig. 5) and cytoplasmic Ca**2+ levels (Fig. 5) suggested that the initial elevation of Ca**2+ may be involved in the translocation of PKC whereas the subsequent decline in Ca**2+ between 15 and 60 s could be involved in terminating the membrane association. These possibilities were examined in this and the following section.

Acute removal of extracellular Ca**2+ by EGTA chelation...
Hormone-stimulated Protein Kinase C Translocation

**FIG. 4.** TRH stimulation of GH₃ cell DAG levels. Duplicate cultures were treated with 1 μM TRH (A) for the indicated times. Untreated cultures (○) were harvested at the beginning and end of the experiment. DAG levels in lipid extracts were determined by a DAG kinase method calibrated using diolein. Mean values are shown with the range of duplicate culture determinations either indicated or within symbol size.

**FIG. 5.** Analysis of cytoplasmic Ca²⁺ levels. Panels A, E, and F were conducted with Fura-2/AM-loaded GH₃ cells. Panels B and C were conducted with cells preloaded with 25 μM Quin-2/AM whereas panel D was conducted with cells preincubated with 500 μM Quin-2/AM. Treatments as indicated by arrows were 1 μM TRH, 50 mM KCl, 0.1 μM ionomycin, or 0.16 μM PMA. Cells in panel C were incubated for 30 min with 100 μM EGTA prior to TRH addition.

(acute E) had no influence on the rapid initial redistribution of PKC stimulated by TRH (Fig. 6A). In contrast, preincubation of cells for 30 min with EGTA (chronic E) to deplete intracellular Ca²⁺ pools resulted in a marked inhibition of TRH-induced PKC redistribution (Fig. 6A). A greater inhibition was observed when Quin-2-loaded cells were preincubated with EGTA (Q+E, Fig. 6A), a condition which decreased TRH-responsive intracellular Ca²⁺ pools as well as cytoplasmic Ca²⁺ levels as shown in Fig. 5C. These results indicated that Ca²⁺ levels were important for hormone-induced PKC redistribution, but it was unclear whether the inhibition resulted from depletion of a TRH-mobilized pool of Ca²⁺ or from a decrease of cytoplasmic Ca²⁺. To distinguish these possibilities, Quin-2 was used as an intracellular chelator at concentrations sufficient to reduce the TRH-stimulated cytoplasmic Ca²⁺ rise without impairing resting cytoplasmic Ca²⁺ levels as shown in Fig. 5D. Quin-2 chelation (Q) was found to reduce the TRH-stimulated PKC redistribution (Fig. 6A). Ionophore treatment of GH₃ cells depletes intracellular Ca²⁺ pools such that TRH no longer stimulates intracellular Ca²⁺ pool mobilization as shown in Fig. 5E. As shown in Fig. 6B, ionomycin or A23187 treatments (I or A) by themselves had no effect on PKC redistribution but blocked the TRH-stimulated redistribution. Conversely, when cytoplasmic Ca²⁺ levels were markedly increased by K⁺ depolarization (Fig. 5A), the TRH-stimulated redistribution of PKC was enhanced (Fig. 6B). These results suggest that the hormone-induced elevation of cytoplasmic Ca²⁺ is required for the initial PKC redistribution.

Since Ca²⁺ depletion inhibited TRH-induced PKC redistribution, we determined whether this treatment interfered with the initial TRH stimulation of DAG production since previous studies indicated that TRH-regulated phospholipid hydrolysis is Ca²⁺-dependent (29). However, as shown in Table I (experiment I), incubation with EGTA had only a small inhibitory effect on TRH-stimulated DAG production whereas ionomycin pretreatment actually enhanced it. Quin-2 loading at high concentrations also had no influence upon the initial increase in DAG (not shown).

The above results indicate that a Ca²⁺ rise per se was insufficient to induce PKC translocation but that the TRH-stimulated Ca²⁺ rise was required for hormone-stimulated PKC translocation.

**TRH-induced PKC Redistribution Is Transient Because of a Limitation in DAG Rather Than Ca²⁺**—It appeared that
Hormone-stimulated Protein Kinase C Translocation

TABLE I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DAG nmol/10^6 cells</th>
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<tr>
<td>None</td>
<td>0.22</td>
</tr>
<tr>
<td>TRH, 20 s</td>
<td>0.37</td>
</tr>
<tr>
<td>TRH with EGTA, 20 s</td>
<td>0.39</td>
</tr>
<tr>
<td>EGTA, 30 min</td>
<td>0.18</td>
</tr>
<tr>
<td>EGTA, 30 min then TRH, 20 s</td>
<td>0.29</td>
</tr>
<tr>
<td>Ionomycin, 30 s</td>
<td>0.21</td>
</tr>
<tr>
<td>Ionomycin, 30 s then TRH, 20 s</td>
<td>0.53</td>
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Experiment II

<table>
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<th>Treatment</th>
<th>DAG nmol/10^6 cells</th>
</tr>
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<tr>
<td>None</td>
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</tr>
<tr>
<td>TRH, 1 min</td>
<td>0.83</td>
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<tr>
<td>TRH, 6 min</td>
<td>1.36</td>
</tr>
<tr>
<td>Ionomycin, 5 min</td>
<td>0.73</td>
</tr>
<tr>
<td>PMA, 5 min</td>
<td>0.87</td>
</tr>
<tr>
<td>TRH, 1 min then ionomycin</td>
<td>1.17</td>
</tr>
<tr>
<td>TRH, 1 min then PMA</td>
<td>1.40</td>
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GHS cells in 6-cm dishes were treated and lipids were extracted for analysis of DAG levels as described under "Experimental Procedures." For experiment I, cultures were treated with 1 \( \mu M \) TRH for 20 s, with 100 \( \mu M \) EGTA for 30 min, or with 0.2 \( \mu M \) ionomycin for 30 s. Where indicated, dishes received sequential treatments of EGTA or ionomycin followed by TRH. For experiment II, cultures were treated with 1 \( \mu M \) TRH for 1 or 6 min, with 10 \( \mu M \) ionomycin for 3 min, with 10 \( \mu M \) PMA for 5 min, or with TRH for 1 min followed by ionomycin or PMA for 5 min. Higher concentrations of ionomycin were used in this experiment in order to promote a large sustained increase in cytoplasmic Ca\(^{2+}\). Duplicate determinations for each treatment differed from each other by less than 5%. 

TRH-stimulated DAG production and Ca\(^{2+}\) mobilization acted in concert to promote the initial redistribution of PKC. Since both TRH-stimulated DAG accumulation and Ca\(^{2+}\) elevation were sustained, we attempted to determine which of these two signals might be relatively limiting especially beyond 1 min of TRH treatment when membrane PKC levels had declined. In these studies, we attempted to stimulate the translocation of PKC in TRH-treated cells either by addition of PMA to mimic a DAG signal or by treatment with ionophores or high K\(^{+}\) to elevate Ca\(^{2+}\).

As shown in Fig. 7A, PMA promoted only a small increase in membrane PKC during brief 5- and 10-min incubations. TRH alone stimulated a typical transient redistribution. In contrast, addition of TRH to PMA-treated cells resulted in a markedly potentiated, biphasic, and sustained stabilization of PKC in the membrane. The biphasic time course of the potentiated response resembled the time course of TRH stimulation of cytoplasmic Ca\(^{2+}\) levels (see Fig. 5A), and it is likely that a TRH-stimulated Ca\(^{2+}\) rise acted to potentiate the effects of PMA. Consistent with this interpretation, we found that EGTA addition reversed the potentiating effect of TRH (see Table I). Ionomycin, or A23187 to further elevate cytoplasmic Ca\(^{2+}\) levels (as in Fig. 5A), there was no observed PKC membrane association beyond that seen with TRH alone (not shown). Such treatments did not markedly alter hormone-stimulated DAG levels (Table I, experiment II) although PMA itself enhanced DAG levels. PMA did not modify the hormone-stimulated Ca\(^{2+}\) elevation (Fig. 5F).

In contrast, when TRH-treated cells were treated with depolarizing K\(^{+}\), ionomycin, or A23187 to further elevate cytoplasmic Ca\(^{2+}\) levels (as in Fig. 5A), there was no observed PKC membrane association beyond that seen with TRH alone (not shown). Such treatments did not markedly alter hormone-stimulated DAG levels (Table I, experiment II). It would appear from these studies that TRH-stimulated DAG rather than Ca\(^{2+}\) is relatively limiting for TRH stimulation of PKC translocation (see "Discussion").

**TRH-stimulated DAG Undergoes a Shift in Composition—**

The results of the preceding studies suggested that DAG rather than Ca\(^{2+}\) may be limiting for maintaining the membrane association of PKC. This conclusion appeared to contradict the observation that TRH stimulated the sustained production of DAG (Fig. 4). Therefore, additional studies were undertaken to analyze the composition of DAG species formed in response to TRH treatment. As shown in Fig. 8, an analysis based on degree of unsaturation indicated that DAG species present within 15 s of TRH addition were almost entirely tetraenoic. In contrast, beginning at 1 min (not shown) and increasing for at least 10 min (Fig. 8), DAGs of trienoic, dienoic, monoenoic, and saturated classes were present. Each of these classes contained only alkali-sensitive species, indicating that alkylacylglycerols were not generated. These results indicate that a significant shift in DAG composition occurs throughout the time course of TRH stimulation. Such a shift in DAG composition may indicate marked alterations in DAG pools associated with PKC activation (see "Discussion").

To further explore the basis for altered DAG regulation of PKC during the time course of TRH stimulation, DAG ac-
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Fig. 8. Analysis of double bond composition of DAG in TRH-treated cells. Cultures were treated with 1 μM TRH for 15 s or 10 min. Lipids were extracted and processed for the DAG kinase assay as indicated to the legend to Fig. 4. [2H]dimethyl Ptd derivatives were analyzed by AgNO₃-thin layer chromatography as described under "Experimental Procedures." The mean and range of duplicate determinations is shown.

Fig. 10. Stimulation of prolactin secretion by TRH and PMA. 3.5-cm cultures of GH₃ cells were incubated at 37 °C without addition (O), with 1 μM TRH (□), with 0.16 μM PMA (△), or with TRH plus PMA (●). Prolactin secreted into the culture medium at indicated times was analyzed by radioimmunoassay as previously described (31, 35). The mean of duplicate determinations which differed by less than 5% is shown.

whether similar amounts of PKC were available for membrane recruitment, maximally effective concentrations of PMA were tested prior to and following TRH treatment. In both cases, similar levels of membrane-associated PKC induced by PMA were detected (Fig. 7C) whereas cytosol fractions were largely depleted of the enzyme (not shown). This result indicates that the transient redistribution of PKC associated by TRH did not result in a desensitization of enzyme molecules to further membrane recruitment.

Biological Significance of PKC Translocation—It has previously been suggested that the sustained stimulation of PRL secretion by TRH may be mediated through the activation of PKC (31). Since combined TRH and PMA treatment resulted in an enhanced membrane stabilization of PKC (Fig. 7, A and B), we assessed the effect of this treatment on PRL secretion as shown in Fig. 10. In a 5-min incubation, TRH (1 μM) or PMA (0.16 μM) alone stimulated PRL release 4- and 8-fold, respectively. The combined treatment with TRH plus PMA resulted in a 20-fold stimulation of PRL release, indicating a marked synergism between these agents.

DISCUSSION

Hormone-stimulated PKC Redistribution Requires Both DAG and Ca⁺⁺ Elevation—Numerous hormonally regulated responses have been found to be synergistically stimulated by activation of PKC (with phorbol diesters or synthetic DAGs) in combination with elevating cytoplasmic Ca⁺⁺ (with ionophores or K⁺ depolarization). PRL secretion by GH₃ cells is stimulated synergistically by Ca⁺⁺ and PKC activation (32-34), and the resultant response resembles that elicited by TRH treatment (31, 34-36). Synergism between Ca⁺⁺ and PKC activators has commonly been interpreted as representing synergism between Ca⁺⁺-activated and PKC-mediated effector pathways (2). However, an alternative basis for synergism is the potentiating effect of a cytoplasmic Ca⁺⁺ rise on DAG activation of PKC.

Wolf et al (5, 37) have suggested that elevated Ca⁺⁺ may serve to "prime" the activation of PKC by promoting chelator-sensitive membrane association of the enzyme without acti-
Desensitization of receptor-associated transduction components is unlikely since TRH-stimulated inositol phosphate formation of the hormone was excluded by readdition studies. The hormone removal in GH3 cells. TRH-stimulated DAG accumulation was transient and reversible upon hormone removal in GH3 cells. TRH-stimulated DAG accumulation was rapid and presumably essential for triggering PKC redistribution. However, TRH-stimulated intracellular Ca2+ mobilization also appeared to be essential for hormone-induced PKC redistribution. Cells preincubated with EGTA or with ionophores failed to exhibit a rise in cytoplasmic Ca2+ in response to TRH due to the depletion of intracellular Ca2+ pools. In addition, buffering loads of Quin-2 inhibited the hormone-induced cytoplasmic Ca2+ rise. Such treatments were found to inhibit TRH-induced PKC membrane association. A similar result for ionomycin pretreatment in inhibiting TRH-stimulated PKC redistribution measured enzymatically has been reported by Fearon and Tashjian (39). In addition, we showed that pretreatment with EGTA or ionomycin did not inhibit the initial TRH-stimulated DAG accumulation. Hence, it appears (a) that TRH-stimulated DAG generation in the absence of a cytoplasmic Ca2+ rise is insufficient, and (b) that a cytoplasmic Ca2+ rise per se is insufficient to promote a stable PKC membrane association. Therefore, TRH-stimulated DAG and Ca2+ are both necessary for the early hormone-stimulated redistribution of PKC.

Possible Bases for the Transient Nature of TRH-induced PKC Redistribution—The primary motivation for the present work was to establish the basis for the transient PKC membrane association observed in TRH-treated cells. Our results rigorously exclude a number of possible explanations. Inactivation of the hormone was excluded by readmission studies. Deensensitization of receptor-associated transduction components is unlikely since TRH-stimulated inositol phosphate generation (40), DAG accumulation (9, 10, and this study), Ca2+ elevations (11, 12), and prolactin secretion (35) are sustained and reversible upon hormone removal in GH3 cells. Down-regulation of PKC by membrane-associated proteolysis (13) was excluded by immunochemical quantitation (Fig. 3B) which indicated that there was complete conservation of PKC during transit between cytosol and membrane during the initial 10-min period of TRH treatment. The rapid termination of a transient cytoplasmic Ca2+ rise could conceivably destabilize the membrane PKC association. However, TRH-stimulated PKC membrane association was stable to isolation in EGTA-containing buffers. Moreover, the sustained Ca2+ elevation promoted by TRH appeared to be adequate to potentiate the membrane recruitment of PKC by PMA (Fig. 7B). Hence, it is unlikely that the decreases in Ca2+ levels between 15 and 60 s following TRH addition causes the precipitous decrease in membrane-associated PKC.

Several additional possible explanations for the transient PKC membrane association were addressed by the experiment of Fig. 7C. PKC is known to undergo autophosphorylation in vitro (14) and appears to alter its substrate specificity in PMA-treated cells (27). Membrane-associated proteins which may interact with PKC may also serve as substrates for the enzyme (15). Hence, it was possible that DAG-triggered activation of PKC stimulated by TRH results in alterations which render PKC incapable of remaining membrane-associated. Our results indicate that, if such modifications do occur, they do not prevent PMA from stimulating the translocation of the entire cellular complement of immunoreactive PKC to the membrane. Kolesnick and Clegg (30) have suggested that DAG-stimulated phosophomycin ceramide formation may result in the production of sphingoid bases which exert an inhibitory effect on PKC and which might serve as a termination mechanism for PKC activation. For DAG, however, the onset of sphingomyelin hydrolysis in GH3 cells was slow compared with the termination of PKC translocation that we observed with TRH. In addition, TRH was not found to elevate ceramide levels in GH3 cells. It has also been suggested that alkylacylglycerols, generated in some cells by receptor activation (55–57), may serve to inhibit PKC following its initial activation (56, 58). However, we were unable to detect alkylacylglycerol formation in response to TRH treatment. Moreover, the results of Fig. 7C argue against the possible secondary activation of inhibitory lipids since PMA was fully effective in recruiting PKC to the membrane in TRH-treated cells. Previous studies in other cell types have indicated that the rapid metabolism of agonist-stimulated DAG may be responsible for transient activation of PKC (2). The present study confirmed previous labeling studies (9, 10) with mass determinations of DAG and found the TRH stimulation of DAG accumulation to be sustained and persist well beyond the period of PKC membrane association. In spite of the sustained elevation of DAG by TRH, the addition of PMA (but not Ca2+ ionophores or high K+) to TRH-treated cells synergistically stimulated a retranslocation of PKC to the membrane (Fig. 7B), suggesting that DAG was relatively limiting for PKC activation by TRH. Therefore, a further analysis of TRH-stimulated DAG species was undertaken. It was found (Fig. 8) that a marked switch in DAG classes occurred throughout the period of hormone stimulation. TRH-stimulated DAG at 15 s was almost entirely tetraenoic whereas DAG species present after 60 s of stimulation consisted of 0–3 double bonds as well as the tetraenoic species. In addition, it was found that plasma membrane DAG was only transiently elevated in response to TRH, exhibiting a time course that approximated that of the membrane association of PKC. These results suggested an explanation for the transient membrane association of PKC.

During the initial 15-s period of TRH stimulation, the mass of DAG (0.2 nmol/106 cells) formed corresponds to the mass of polyphosphoinositides hydrolyzed by phospholipase C (9). The DAG formed was almost entirely stearoyl arachidonyl glycerol, a species which predominates in the polyphosphoinositides of GH3 cells (41). We propose that the polyphosphoinositide-derived DAG formed initially in a focal region of the plasma membrane is responsible for PKC translocation and that the PKC membrane association is terminated upon metabolism of this DAG to Ptd. MacDonald et al. (42) have reported that the membrane-associated DAG kinase exhibits a strong preference for stearoyl arachidonyl glycerol. It is possible that this enzyme is compartmentalized within or translocated to (43) a region of the plasma membrane which also includes hormone receptors, hormone-responsive polyphosphoinositide pools, and phospholipase C. Initial PKC

4 T. F. J. Martin, unpublished results.
translocation would be predicted to occur in this focal region of the plasma membrane.

In contrast to the initially formed DAG, the species formed during the sustained stimulation by TRH probably derive from phospholipids other than polyphosphoinositides, from enzymatic routes other than the receptor-regulated polyphosphoinositide-specific phospholipase C, and may be present largely in intracellular membranes. Consistent with this conclusion, amounts of DAG (1 nmol/10^6 cells) which exceed the mass of polyphosphoinositides are formed at later times. Imai and Goroshengorn (45) have proposed that there is a shift from PtdIns 4,5-P_2 to PtdIns hydrolysis in TRH-stimulated GH_2 cells. Our results are partially consistent with this suggestion, however, it is unlikely that all of the DAG formed at late times is PtdIns-derived since PtdIns levels (1-2 nmol/10^6 cells) are reported to decrease not at all (9, 41) or by less than 20% (44) at 10 min of TRH stimulation. In addition, saturated DAGs generated during the sustained response to TRH likely arise from the hydrolysis of phosphatidylincholine, the sole phospholipid in GH_2 cells which contains saturated DAGs. Proposed alternative routes for stimulated DAG production include sustained nonpolyphosphoinositide phospholipid hydrolysis by a Ca^{2+}-activated phospholipase C (46) or phosphatidylcholine hydrolysis by phospholipase C (47) or D (48) mechanisms. These alternative pathways may be second messenger-regulated (49). We have found that DAG levels are modestly increased in GH_2 cells by PMA or Ca^{2+} ionophore treatment (Table I), suggesting second messenger activation of an alternative enzymatic route leading to sustained DAG production.

It has previously been suggested that DAG derived from phosphatidylincholine hydrolysis may be capable of activating PKC (50). Moreover, _in vitro_ studies indicate that most classes of DAG are capable of activating PKC except for saturated species (51-53). From these observations, it might be predicted that membrane bilayer concentrations of DAG, possibly formed in cellular compartments other than the plasma membrane, are insufficiently high to activate PKC or that the membrane PKC association formed with these other DAG species does not survive the cellular extraction procedures that we have used. If the first alternative is correct, it would suggest that the secondary formation of DAG stimulated by TRH may be involved in the regulation of cellular processes which do not involve PKC.

**Significance of TRH-induced PKC Translocation for TRH-regulated Responses**—TRH stimulation of PRL secretion in GH_2 cells is biphasic with an initial transient phase followed by a sustained phase. Activation of PKC has previously been implicated in the sustained secretory response (31, 34-36). However, PKC activation as detected by membrane stabilization of the enzyme is transient. Such observations imply that the sustained secretory response to PKC activation is mediated by a stable change in protein phosphorylation which outlasts the transient phase of PKC activation. PKC-mediated changes in protein phosphorylation in response to TRH are sustained (54). However, the magnitude of the sustained secretory response may be influenced by the extent of the initial activation of PKC. Such a view is consistent with the observation that treatment with TRH and PMA results in a potentiateg membrane stabilization of PKC and a potentiated stimulation of PRL secretion. Since the sustained secretory response to TRH is readily reversed upon hormone removal (35), components other than DAG and PKC (e.g. cytoplasmic Ca^{2+} levels), whose levels are determined by continuous receptor occupancy, must be involved in maintaining the secretory response.

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**REFERENCES**

Hormone-stimulated Protein Kinase C Translocation

The sustained second phase of hormone-stimulated diacylglycerol accumulation does not activate protein kinase C in GH3 cells.
T F Martin, K P Hsieh and B W Porter


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