Phosphorylation Substrates for Protein Kinase C in Intact Pituitary Cells: Characterization of a Receptor-Mediated Event Using Novel Gonadotropin-Releasing Hormone Analogues

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ABSTRACT: The involvement of protein kinase C in the signal transduction of gonadotropin-releasing hormone (GnRH) action was investigated with a GnRH superagonist, partial agonists, and antagonists in intact rat pituitary cells. Exposure of ^{32}P-labeled cells to GnRH or to the superagonist [D-Nal(2)^6]GnRH (200 times GnRH potency in vivo) induced the enhanced phosphorylation of 42-, 34-, 11-, and 10-kDa proteins and the dephosphorylation of a 15-kDa protein as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/autoradiography. This effect was blocked in a dose-dependent manner by potent GnRH antagonists. At its maximally effective concentration of 10^{-9} M, [D-Nal(2)^6]GnRH induced an up to 2 times more pronounced phosphorylation of endogenous substrates than GnRH at 10^{-7} M. This was in accord with its ability to cause an 8-fold increase in the translocation of protein kinase C to the particulate fraction vs. 3.4-fold for GnRH. This effect correlated with potency for a series of GnRH agonists ([D-Nal(2)^6]GnRH > GnRH > [Gly^2]LH-RH) and was prevented by GnRH antagonists, as assessed by a novel phorbol ester receptor binding assay and by a standard kinase assay. Downregulation of protein kinase C by prolonged incubation of the pituitary cells with high concentrations of active phorbol esters abolished protein kinase C activity and also prevented the phosphorylation induced by GnRH, or [D-Nal(2)^6]GnRH. The same effect was obtained by preincubating the cells with the protein kinase C inhibitor H-7. In this study we identify for the first time physiological substrates for protein kinase C in intact pituitary cells. We demonstrate a close quantitative correlation between the extent of translocation of protein kinase C, levels of phosphorylation of specific substrates in the intact cells, and the biological activity of the GnRH analogues with varying affinity for the GnRH receptor. These data strengthen the contention that the physiological effects of GnRH are primarily mediated via the phosphatidylinositol/Ca^{2+} signal transfer system and represent a first step toward defining the physiological substrates of protein kinase C and their role in the cascade of events that starts upon binding of GnRH to its receptor.

Gonadotropin-releasing hormone (GnRH)[1] is a hypothalamic decapeptide that acts as a releasing hormone to regulate pituitary gonadotropin secretion (Conn et al., 1985). However, the sequence of biochemical events leading from pituitary cell receptor occupancy to LH and FSH secretion remains obscure. Most studies have concentrated on the role of Ca^{2+} as a mediator of GnRH biological action (Conn et al., 1984). Thus, drugs that elevate intracellular Ca^{2+} levels can induce gonadotropin release with the same efficacy as GnRH; conversely, gonadotropin release in response to GnRH is terminated virtually immediately following removal of extracellular Ca^{2+}.

Recently, it has been demonstrated that GnRH stimulates polyphosphoinositide hydrolysis in rat pituitary gonadotrophic cells (Kiesel & Catt, 1982; Kiesel et al., 1986) yielding 1,2-diacyl-*sn*-glycerol (DAG) and inositol triphosphate. In a variety of cell types, DAG activates the Ca^{2+}-activated/phospholipid-dependent protein kinase (protein kinase C) (Niedel et al., 1983; Nishizuka, 1984; Hannun et al., 1986) by a process thought to involve in many instances translocation of the enzyme from the cytosol to the membrane (Anderson et al., 1985; Drust & Martin, 1985; Fearon & Tashjian, 1985). In gonadotrophic cells, phorbol esters and synthetic diacylglycerols, which occupy the same binding site on protein kinase C as DAG, stimulate LH secretion (Negro-Vilar & Lapetina, 1985; Conn et al., 1985a,b) by a mechanism independent of extracellular Ca^{2+}. Taken together, these findings suggest that phosphorylation of endogenous substrates by protein kinase C is necessary for release of LH/FSH by GnRH.

In order to understand the role of protein kinase C in signal transduction, an important step is to identify its substrates for phosphorylation in an intact cell system. In this study, we used an array of GnRH analogues (agonist, partial agonist, antagonist) of varying potency to characterize the protein kinase C substrates in intact pituitary cells. We developed a convenient phorbol ester receptor binding assay to monitor the number of binding sites in the particulate vs. cytosolic fractions of the treated cells. Our results indicate that there is a direct correlation between the ability of these GnRH analogues to promote translocation of the phorbol ester binding sites from the cytosol to the particulate fraction of the treated cells, the

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[1] Abbreviations: PKC, calcium-activated/phospholipid-dependent protein kinase; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; [^{3}H]PDBu, [^{3}H]phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; diC$_8$, 1,2-dioctanoyl-*sn*-glycerol; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; IP$_3$, D-*myo*-inositol 1,4,5-triphosphate; PI, phosphatidylinositol; DAG, diacylglycerol; PS, phosphatidylserine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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extent of phosphorylation of specific substrates in situ, and their biological activity.

Experimental Procedures

Materials

$^{32}PO_4$ and [^{3}H]PDBu were from Du Pont/New England Nuclear. Phosphatidylserine and PMA were purchased from Sigma. diC_8 was from Avanti. H-7 was a generous gift from Takayoshi Kida, Seikagaku America, Inc. DEAE-cellulose (DE-52) was obtained from Whatman. Electrophoresis reagents were from Bio-Rad Laboratories. Premixed low molecular weight standards were from Pharmacia. All other compounds were of reagent grade.

Methods

Preparation of Dispersed Rat Pituitary Cells. The 18–20 day old male Sprague-Dawley rats were anesthetized by CO_2 inhalation and euthanized by decapitation. Anterior pituitaries were removed, and the cells were dispersed as previously described (Vale et al., 1972) with the following minor modifications. Briefly, anterior pituitary fragments were placed in 10 mL of Dulbecco's modified Eagle's medium (DMEM) containing 0.2% trypsin and 0.1 mg of DNase and incubated with rocking at 37 °C for 35 min. Residual tissue fragments were washed 3 times with DMEM followed by three washes with 0.1% soybean trypsin inhibitor. After four additional washes, the cells were resuspended at 3×10^6 cells/mL in DMEM containing 10% fresh rat serum and 10% heat-inactivated fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and L-Glutamine (2 mM), distributed to six-well dishes (3×10^6 cells/well), and cultured at 37 °C in humidified air with 5% CO_2 for 3 days.

$^{32}PO_4$ Incorporation. Prior to labeling, cultures were rinsed with serum-free, phosphate-free HEPES-buffered (25 mM, pH 7.5) DMEM. One milliliter of the same medium containing 0.1 mCi/mL $^{32}PO_4$ was added for 4 h at 37 °C. At the end of the 4-h labeling period, cells were washed 4 times, agents were added for indicated times, and finally cells were washed with ice-cold medium and quickly frozen in liquid N_2. Subsequently, the frozen cells were thawed in 0.3–0.5 mL of ice cold "lysis buffer" (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 100 mM NaF, 0.5 mM DL-dithiothreitol, 0.1 mM PMSF, 0.25 M sucrose), scraped with a rubber policeman, and homogenized (70 up-and-down strokes on ice) in a tight-fitting Dounce homogenizer. The homogenates were centrifuged at 400*g* for 10 min at 4 °C, and the supernatant was centrifuged at 160000*g* for 5 min at 4 °C in a Beckman Airfuge using a 30° A-100 rotor. The cytosolic supernatant and pellet were separately dissolved in sodium dodecyl sulfate (SDS) sample buffer (25 mM Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 5% β-mercaptoethanol) and boiled for 5 min. Polyacrylamide slab gel electrophoresis was carried out according to the method of Laemmli (1970), and autoradiographs were prepared. The relative intensity of each band was quantitated by densitometric tracing with a Zeineh soft laser scanning densitometer (Model SLR, Biomed Instruments, Inc., Fullerton, CA) and the Zeineh Videophoresis II software program for the Apple II computer.

Protein Kinase C Assay. Pituitary cells were incubated for 5 min with various GnRH analogues. At the end of the incubation, cells were washed twice with cold PBS. Subsequently, the PBS was removed by aspiration, and the cells were lysed in 0.3 mL of ice cold lysis buffer. The cytosolic supernatant was prepared as described above. Protein kinase C activity was quantitated by measuring incorporation of ^{32}P from [γ-^{32}P]ATP into histone type H1 essentially as described (Niedel et al., 1983), with the following modifications: the reactions were carried out for 3 min at 30 °C, and the TCA-precipitated protein was collected and washed on Whatman GF/C glass fiber filters under vacuum with a cell harvester (Syntex Corp., Palo Alto, CA).

Downregulation of Phorbol Diester Receptors. Dispersed rat pituitary cells were incubated with 10^{-6} M PMA in DMEM with 1% (w/v) bovine serum albumin (BSA) for 30 h essentially as described Collins & Rozengurt, 1982a,b; Rozengurt et al., 1983). To demonstrate that this preincubation with phorbol esters made the cells effectively protein kinase C deficient, we measured the number of phorbol ester binding sites in intact cells as well as in the cytosol and pellet extract from the high-speed centrifugation (160000*g*, 5 min).

Phorbol Ester Receptor Binding Assay. Pituitary cells were lysed in lysis buffer and homogenized in a tight-fitting Dounce homogenizer. After a low-speed spin (400*g*, 10 min), the supernatant was centrifuged at 160000*g* for 5 min. The high-speed pellet was extracted with 0.2% Triton X-100 in lysis buffer for 1 h on ice with continuous stirring, followed by centrifugation at 160000*g* for 5 min. This supernatant and the cytosol isolated after the first 160000*g* sedimentation were used to measure [^{3}H]PDBu binding sites. Briefly, the binding assay was carried out in a mixture of 20 μg/mL PS (dried under N_2 and sonicated in 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1% BSA) and 160 nM [^{3}H]PDBu to which was added 50 μL of cytosol or pellet extract in a buffer containing 20 mM Tris-HCl, pH 7.5, and 1.6 mM $CaCl_2$ in a reaction volume of 0.25 mL. Identical results are obtained with 1% Triton X-100 mixed micelles containing 15 mol % PS (Hannun & Bell, 1986). Nonspecific binding was defined as [^{3}H]PDBu binding in the presence of 5 μM PMA. After a 15-min incubation at 22 °C in a shaking bath, 1 mL of ice cold wash buffer (20 mM Tris-HCl, pH 7.5, 10% methanol) was added, and bound radioactivity was separated from "free" by five washes on a DE-82 filter, with a PHD cell harvester Model 200A (Cambridge Technology, Inc., Cambridge, MA).

Protein was measured according to the method of Bradford (1976). BSA was used as the standard.

Peptide Synthesis. Peptides were synthesized as described (Nestor et al., 1982, 1984) by the solid-phase method on benzhydrylamino–1% divinylbenzene–polystyrene beads (0.6 mmol/g; Peninsula Laboratories) with N^{α}-(*tert*-butyloxycarbonyl)-protected amino acids. After deprotection/cleavage in 90% HF/anisole (1 h, 0 °C), the peptide was washed on a fitted glass funnel with diethyl ether (3×) and dissolved in acetic acid. The crude peptide was passed through a short column of Bio-Rex AG-3 (acetate form) to replace the F counterion and lyophilized. The crude peptide was purified in 200-mg batches by preparative reversed-phase high-performance liquid chromatography (2.5 × 100 cm column of Vydac TP-218, 20 μm) as described (Nestor et al., 1984) with as eluent acetonitrile (~30% for agonists; ~60% for RS-29226 and RS-68439) in water (eluent was 0.03 M in ammonium acetate, pH 4.5). The appropriate fractions were pooled, concentrated, and lyophilized.

The analogues studied were as follows: RS-94991 ([D-Nal(2)^6]GnRH), a superagonist (200 times GnRH potency in vivo) (Nestor et al., 1982); [Gly^2]GnRH, a partial agonist (Nestor et al., 1984); des-His^2-GnRH, a low-affinity pure antagonist (Vale et al., 1972b); RS-29226 ([*N*-Ac-Pro^1,D-pCl-Phe^2,D-Nal(2)^{3,6}]GnRH), a high-affinity antagonist (Nestor et al., 1982); RS-68439 ([*N*-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-Arg(Et$_2$)^6,D-Ala^{10}]GnRH), a highly potent and long-acting antagonist (Nestor et al., 1983). The abbreviations

Table I: Effect of GnRH Antagonists on GnRH-Induced ^{32}P Incorporation into Pituitary Proteins

treatment	concn of antagonist (M)	percent [^{32}P]P_i incorporated				
		M_r 42000	M_r 34000	M_r 15000	M_r 11000	M_r 10000
no treatment		10 ● 1.5	ND	100 ± 12	ND	ND
GnRH		100 ± 13	100 ± 12	50 ± 7	100 ± 9	100 ± 14
GnRH + RS-29226	10^{-9}	96 ± 15	87 ± 21	46 ± 16	50 ± 12	80 ± 25
GnRH + RS-29226	10^{-8}	35 ± 5	40 ± 6	80 ± 10	ND	ND
GnRH + RS-29226	10^{-7}	15 ± 0.9	10 ± 2	100 ± 7.8	10 ± 1.6	ND
GnRH + RS-68439	10^{-9}	25 ± 6	15 ± 4	74 ± 12	18 ± 3	30 ± 6
GnRH + RS-68439	10^{-8}	7 ± 4	ND	100 ± 21	ND	ND
GnRH + RS-68439	10^{-7}	ND	ND	96 ± 12	ND	ND

[a] ^{32}P-Labeled pituitary cells were preincubated with RS-29229 or RS-68439 for 1 h and challenged with GnRH (10^{-7} M) for 10 min. Quantitation of protein phosphorylation was performed by densitometric scanning of the autoradiographs. Results are expressed as percentages of the ^{32}P incorporated into each protein in the presence of 10^{-7} M GnRH except for the M_r 15000 protein, which was maximally phosphorylated in control cells. Values represent the mean of data from three separate experiments ± SE. ND = not detectable by scan densitometry.

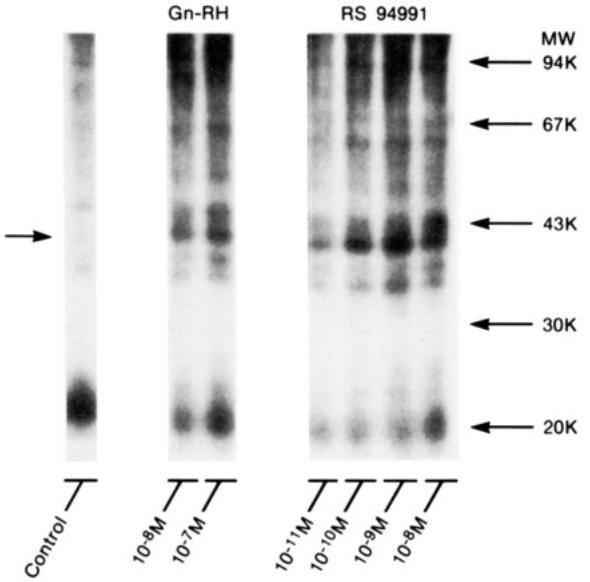

FIGURE 1: Dose response of GnRH and RS-94991 on protein phosphorylation in intact rat pituitary cells. Autoradiogram of SDS–PAGE (10%) of ^{32}P-labeled proteins in the 160000*g* pellet. Indicated concentrations of the agents were added for 10 min. Molecular weight standards (Pharmacia) are phosphorylase (M_r 9400, albumin (M_r 67000), ovalbumin (M_r 43000), and carbonic anhydrase (M_r 20000).

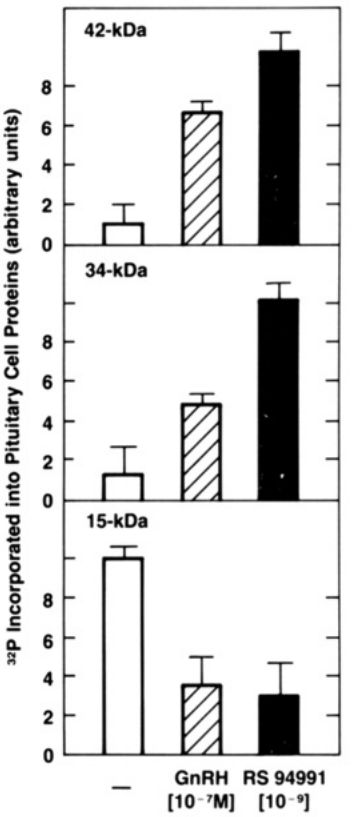

FIGURE 2: Effect of GnRH and RS-94991 on the phosphorylation status of the 42-, 34-, and 15-kDa proteins in intact pituitary cells. GnRH (10^{-7} M) and RS-94991 (10^{-9} M) were present in the culture for 10 min. ^{32}P incorporation into the substrates was determined by densitometric analysis of the resultant autoradiograms and is expressed in the form of arbitrary units. Each value represents the mean of three separate experimental determinations (±SE).

for the unnatural amino acids used are as follows: Nal(2), 3-(2-naphthyl)alanine; pCl-Phe, *p*-chlorophenylalanine; hArg(Et_2), N^G,$N^{G'}$-diethylhomoarginine.

Results

Treatment of intact pituitary cells with GnRH for 10 min induces the phosphorylation of several endogenous proteins of approximate molecular masses of 42- and 34-kDa in the microsomal pellet fraction of ^{32}P-labeled cells as analyzed by one-dimensional SDS–PAGE (Figure 1). In addition, a 15-kDa protein was partially dephosphorylated. The effect was stable over a 5–30-min time period (not shown). An analogue of GnRH with 200 times the GnRH potency, [D-Nal(2)^6]-GnRH (RS-94991) (Nestor et al., 1982), exhibits similar effects on protein phosphorylation in intact pituitary cells in a dose-dependent manner. However, by quantitating the extent of phosphorylation of the substrate proteins in response to increasing concentration of GnRH or RS-94991, it was found that RS-94991 was as effective as GnRH at a 100-fold lower concentration. Moreover, at their maximally effective concentrations (10^{-9} M for RS-94991 vs. 10^{-7} M for GnRH), the superagonist induced greater phosphorylation of the 42- and 34-kDa proteins (1.5–2-fold increase over the effect of GnRH). The dephosphorylation of the 15-kDa protein appeared to occur to the same extent with either agent (Figure 2).

In order to assess whether this effect of GnRH and RS-94991 was a receptor-mediated event, intact pituitary cells were preincubated for 1 h with the specific GnRH antagonist RS-29226 (Nestor et al., 1982), followed by a 10-min challenge with GnRH. The phosphorylated proteins in the microsomal pellet fraction were subjected to a 6–15% SDS–PAGE gradient to obtain a better resolution of the phosphorylated bands. Using these conditions, we detected two more phosphorylated proteins: 11 and 10 kDa. Figure 3 and Table I show that preincubation of pituitary cells with 10^{-9}–10^{-7} M RS-29226 blocked the effect of 10^{-7} M GnRH in a dose-dependent manner. Preincubation of the pituitary cells with RS-68439, followed by challenge with GnRH, had a more dramatic effect than RS-29226 (Table I), demonstrating its greater potency. These GnRH antagonists were also tested

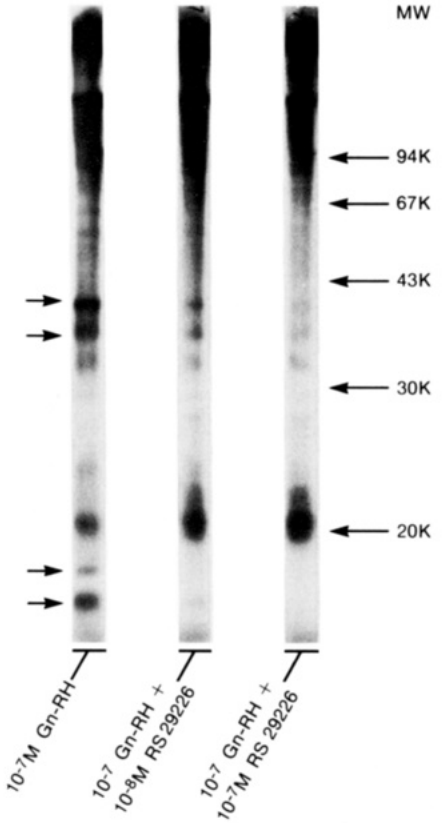

FIGURE 3: Inhibition of GnRH-induced phosphorylation patterns in intact rat pituitary cells by GnRH antagonist RS-29226. ^{32}P-Labeled cells were incubated with RS-29226 at the indicated concentrations for 1 h, followed by 10-min challenge with GnRH. Cells were fractionated as described under Experimental Procedure; 160000*g* pellets were subjected to 0.1% SDS–6–15% polyacrylamide gel electrophoresis, followed by autoradiography. A representative experiment is shown. The experiment was repeated 3 times with comparable results.

against the superagonist (10^{-9} M) with similar results (not shown).

To assess whether the effect of GnRH is mediated via protein kinase C stimulation, we incubated the cells with agents that bind directly to and stimulate the enzyme. Active phorbol esters (PMA, 10^{-7} M) and synthetic diacylglycerols (diC_8, 5 × 10^{-5} M) mimic the effect of GnRH, while the inactive phorbol analogue 4$^{\alpha}$-methyl-TPA was unable to induce this effect (data not shown).

GnRH activation of gonadotropin release is associated with subcellular redistribution of protein kinase C activity from the cytosol to the particulate fractions (Naor et al., 1985; Hirota et al., 1985). This seems to be a physiological effect of GnRH, and it is followed by LH release. In order to compare the effects of various classes of GnRH analogues (superagonist, agonist, partial agonist, and antagonist) of varying potency, on the subcellular distribution of the enzyme, pituitary cells were incubated with each agent for 5 min. The experiment in Figure 4 compares the ability of the different analogues to promote translocation of protein kinase C activity from the cytosol to the particulate fraction of treated cells. For technical reasons, we initially used the disappearance of protein kinase C activity from the cytosol rather than the appearance in the membrane as a measure of translocation. Most (75%) of the total activity exists in the cytosolic fraction of unstimulated cells (Naor et al., 1985; Hirota et al., 1985; present data). It is clear from Figure 4 that the ability to initiate translocation in the pituitary cells varies markedly and in a dose-dependent manner between the various analogues. Preincubation of the

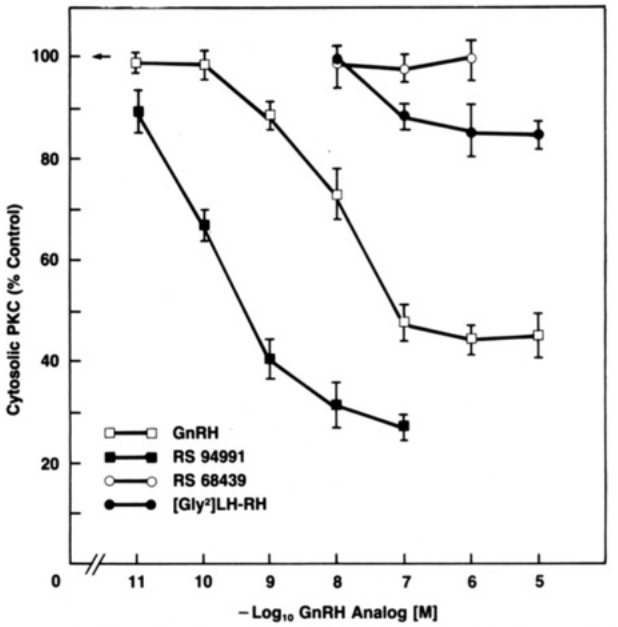

FIGURE 4: Translocation of protein kinase C activity by GnRH analogues. Pituitary cells were prepared as described under Experimental Procedures and incubated for 5 min with or without the indicated concentrations of GnRH analogues. Cytosolic extracts were prepared, and total protein kinase C activity was determined. Results represent the means of quadruplicate determinations ± SE (n = 2). Control values represent the PKC activity found in the cytosol of untreated cells (arrow).

cells with the GnRH antagonist RS-68439 prevented the effect of GnRH or RS-94991 (not shown) and did not have an effect on its own (Figure 4).

In order to overcome the technical problems related to the measurement of protein kinase C activity in the particulate fraction of the cells, we developed a simple receptor binding assay for the assessment of the number of phorbol ester binding sites. This was based on the finding that the protein kinase C behaves as a receptor for phorbol esters with both activities copurifying (Niedel et al., 1983). This assay does not require a purification step (e.g. DEAE column chromatography; Niedel et al., 1983) and measures specific phorbol ester binding sites. Nonspecific binding is 5% for the cytosolic enzyme and 15–20% for the extracted enzyme from the high-speed pellet. The preparation of the subcellular fractions was performed in the presence of EDTA/EGTA to exclude a possible effect of Ca^{2+} on the translocation process (McArdle & Conn, 1986). In the experiment shown in Figure 5, we tested one dose of the GnRH analogues to assess the translocation of the protein kinase C from the cytosol to the particulate compartment of the treated cells by measuring the number of phorbol ester binding sites in both compartments. The results from this assay confirm the earlier finding that GnRH promotes translocation of protein kinase C from the cytosolic compartment (Hirota et al., 1985; Naor et al., 1985; Figure 4). The basic observations on the hierarchy of translocation efficiency of the GnRH analogues were confirmed both for the disappearance from the cytosolic fraction (complete dose responses) (Figure 4) and for the apperance in the membrane fraction (Figure 5) with more limited experiments using [^{3}H]PDBu binding studies. This potency of the translocation hierarchy correlates with the ability of the activated protein kinase C to phosphorylate endogenous substrates in the intact pituitary cells (Figures 1–3). In another series of experiments (n = 2) using pituitary cell cultures from older animals (38 days and 3 months), we have found that the superagonist induces a much smaller translocation effect than the 18–20

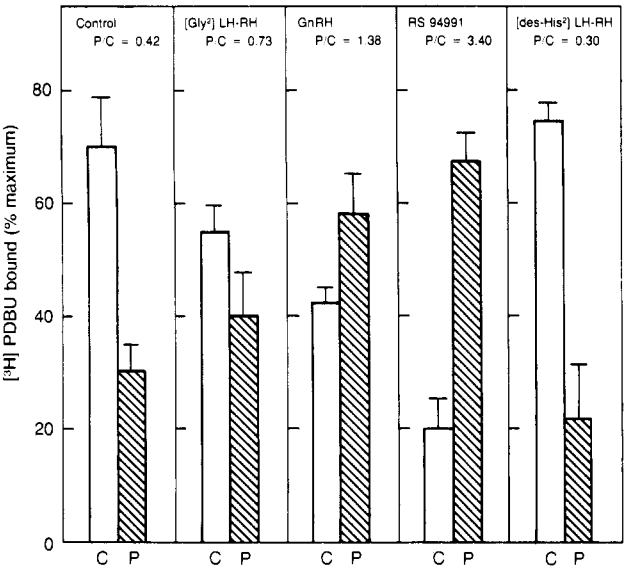

FIGURE 5: Total phorbol diester receptors in the cytosolic (C) and particulate (P) fractions from control and GnRH or GnRH analogue treated pituitary cells. Cells were incubated with GnRH (10^{-7} M), RS-94991 (10^{-9} M), [Gly^2]LH-RH (10^{-7} M), and des-His^2-LH-RH (10^{-7} M) for 5 min. The preparation of the cytosolic and particulate fractions as well as the binding assay using [^{3}H]PDBu was as described under Experimental Procedures. Nonspecific binding determined in the presence of 5 μM PMA has been subtracted from the total binding to yield specific [^{3}H]PDBu binding, which is shown. The data are the mean of triplicate determinations ± SD. The experiment was repeated twice with comparable results. The ratio P/C was calculated by dividing the number of receptors found in the particulate fraction (P) by that found in the cytosol (C) of control vs. treated cells.

day old group, as assessed by both [^{3}H]PDBu binding and kinase activity (data not shown). Work from other groups (Chan et al., 1981; Clayton & Catt, 1981) has indicated that the concentration of pituitary GnRH receptors increases in early life to a maximum between 15 and 30 days of age, regardless of sex. This correlates with higher serum LH levels. In addition, recent work by Conn's group (Conn et al, 1985a,b; Harris et al., 1985) on PKC mediation of GnRH-induced LH secretion was performed on pituitary cells from 22 day old female rats.

To further assess whether the enhanced phosphorylation was indeed due to protein kinase C stimulation, we used two independent approaches: (1) a protein kinase C inhibitor; (2) "protein kinase C downregulation", which renders the cells protein kinase C deficient. Table II shows that preincubation of the pituitary cells with H-7 (5×10^{-5} M) for 2 min followed by challenge with either GnRH or diC$_8$ prevented to a large extent the ability of both agents to induce the phosphorylation of the 42-kDa protein (80–90% inhibition). The other substrates were affected to the same extent (not shown). Exposure of the dispersed rat pituitary cells to 10^{-6} M PMA for 30 h followed by washing decreased the number of phorbol ester binding sites (protein kinase C; Niedel et al., 1983) from 360 fmol/mg in the control cells to undetectable levels in the PMA-exposed cells. These PMA-pretreated cells became unresponsive to GnRH or PMA stimulation (Table II).

Table II: Effect of PKC Downregulation or Pretreatment with H-7

pretreatment	treatment	percent [^{32}P]P$_i$ incorporation into the 42-kDa protein
expt I		
none	GnRH	100 ± 9
none	diC$_8$	91 ± 6
PMA (10^{-6} M)	GnRH	ND
PMA (10^{-6} M)	diC$_8$	ND
expt II		
none	GnRH	100 ± 12
none	diC$_8$	88 ± 11
H-7 (5×10^{-5} M)	GnRH	16 ± 3
H-7 (5×10^{-5} M)	diC$_8$	11 ± 2

[a] GnRH/diC$_8$-stimulated phosphorylation. Downregulation of PKC was accomplished as described under Methods. ^{32}P-Labeled pituitary cells were preincubated with H-7 for 5 min before the addition of stimulants for an additional 10 min. The data presented were calculated from densitometric scan readings of autoradiograms (±SE; n = 3). ND = not detectable.

Discussion

An extensive series of analogue synthesis programs have resulted in three classes of GnRH analogues: "superagonists" with in vivo potencies 200 or more times that of GnRH (e.g., RS-94991; Vickery et al., 1985) and receptor-binding affinities greater than GnRH itself; partial agonists with reduced intrinsic agonistic activity (e.g., [Gly^2]GnRH; pure antagonists (RS-29226, RS-68439, des-His^2-GnRH), some with receptor binding affinities greater than GnRH (McRae et al., 1984). These analogue classes offer probes that assist in dissecting the components of the intracellular second messenger systems involved in GnRH-stimulated LH and FSH release.

The observation that phorbol esters and synthetic diacylglycerols can stimulate LH release (Negro-Vilar & Lapetina, 1985; Conn et al., 1985a,b) suggests that protein kinase C activation can be asociated with the LH release process. In an effort to explore this process further, we have used a panel of GnRH analogues with a hierarchy of effects on LH secretion (Nestor et al., 1982, 1984) and compared these to their effects on protein kinase C translocation and phosphorylation of substrates in situ. Our results indicate that the ability of these analogues to translocate enzyme activity from the soluble to the particulate compartment of the cell correlates well with their ability to stimulate substrate phosphorylation and ultimately LH secretion.

Some of the PKC substrates described here are of low molecular weight. The following points suggest that they are not the result of proteolytic degradation of a larger molecular weight substrate. The ratio of the phosphorylated proteins (42, 34, 15, 11, and 10 kDa) was constant within each experiment. The lower molecular weight proteins did not accumulate at the expense of the larger molecular weight substrates. The experiments were repeated many times with little variation in the relative proportion of the phosphorylated proteins.

In addition to enhanced phosphorylation, PKC activation is associated with a decrease in the extent of phosphorylation of the 15-kDa protein. We interpret this result as suggesting that the PKC activation induces the rapid activation (via phosphorylation) of a phosphatase that in turn dephosphorylates the 15-kDa substrate. This is an indirect readout for PKC activation and needs to be further investigated.

The earliest leads in the search for antagonists of GnRH were des-His^2-GnRH and [Gly^2]GnRH. From in vitro LH secretion studies it was reported that [Gly^2]GnRH was a very weak partial agonist while des-His^2-GnRH was a low-affinity pure antagonist (Vale et al., 1972b). Figures 4 and 5 show that the weak partial agonist activity of [Gly^2]GnRH is clearly reflected in its ability to promote the translocation of a small but statistically significant number of phorbol binding sites from the cytosol to the particulate fraction of the pituitary cells. In contrast, the weak pure antagonist des-His^2-GnRH was completely inactive in this respect. This demonstrates both the close correlation between the extent of activation of protein

kinase C and the biological potency of these analogues and the validity of the methods employed by us (phosphorylation of endogenous substrates for protein kinase C via a receptor-mediated pathway and translocation of the enzyme) to assess this correlation.

The experiments using the superagonist (RS-94991) (Figures 1, 2, 4, and 5) yielded interesting and somewhat unexpected results. The dose response for phosphorylation was shifted 100 × to the left, and at its maximal concentration, this agent induced a larger degree of translocation of protein kinase C and phosphorylation of substrates than did GnRH. This result cannot be explained merely by the 7-fold receptor affinity of this analogue relative to GnRH (Clayton & Catt, 1981). We suggest that the higher intrinsic activity of the superagonist reflects a more efficient coupling between the superagonist–occupied receptor and the PI turnover pathway. This will result in higher levels of second messengers produced (diacylglycerol and IP_3) and an enhanced activation of the cellular responses dependent upon these effector molecules. These data would suggest the existence of an amplification cascade at the level of the receptor/second messenger system.

Recently, it has been demonstrated that incubation of rat testicular interstitial cells with the superagonist [D-Ala^6,Pro^{9}NHEt]GnRH resulted in increased incorporation of [^{32}P]P_i into PI (Molcho et al., 1984).

Three independent lines of evidence indicate that the enhanced phosphorylation induced by GnRH and the potent analogue RS-94991 is mediated by protein kinase C activation. Agents that stimulate the enzyme directly, bypassing the receptor-mediated pathway (PMA, diC_8), stimulate the phosphorylation of the same proteins as GnRH or the superagonist RS-94991. Treatment with a protein kinase C antagonist blocks the ability of GnRH or diC_8 to stimulate the phosphorylation of these substrates. Downregulation of the enzyme, which renders the cells protein kinase C deficient (Collins & Rozengurt, 1982a,b; Rozengurt et al., 1983; Blackshear et al., 1985; present study), totally prevented the ability of GnRH, RS-94991, PMA, or diC_8 to phosphorylate/dephosphorylate the substrate proteins.

These data do not explain, however, the mechanism by which protein kinase C might interact with the other regulatory pathways of the GnRH action, such as the calcium–calmodulin system. Data from other laboratories (Conn et al., 1972; Turgeon & Waring, 1986) demonstrate that the simultaneous presence of A23187 and PMA resulted in a synergistic response that mimicked the full physiological response of GnRH. This suggests that the stimulatory effect of GnRH and LH release may be mediated by two intracellular pathways involving Ca^{2+} and DAG as second messengers. It has been suggested (Rasmussen & Barrett, 1984) that each of these two messengers may have a unique temporal role: the IP_3/calcium system being responsible for an initial transient cellular response and the DAG/PKC system being responsible for a sustained phase of the cellular response to various hormonal stimuli. Recently, it has been demonstrated that activation of pituitary protein kinase C with PMA results in an amplification of GnRH-induced LH secretion (Turgeon & Waring, 1986).

Understanding the role of protein kinase C in signal transduction requires, as an initial step, identification of its substrates for phosphorylation in an intact cell system. Here we identify these substrates by first activating the cells via a receptor-mediated pathway.

Earlier work (Naor et al., 1985) described several endogenous phosphoproteins that were labeled by protein kinase C in the pituitary. However, these experiments were performed in a cell-free system, with partially purified protein kinase C and pituitary cytosol as the substrate. The physiological relevance of these results might be questioned, since the enzyme was not activated in an intact cell system, nor was it translocated to a new cellular compartment. In addition, several cellular components were missing from the assay. Our present results demonstrate the following: (a) In cells physiologically activated by a ligand via a receptor-mediated pathway, protein kinase C became translocated in a dose-dependent manner. (b) Only the translocated enzyme was able to find the physiological substrates for phosphorylation—these substrates were not available to the enzyme in the "control" cells nor in cells treated with GnRH antagonists (Figures 1, 2, 4, and 5). (c) These substrates were identified in the same postmitochondrial fraction that the enzyme was translocated to. Thus, here we demonstrate the existence of physiological substrates for protein kinase C in a physiologically relevant system: the intact pituitary cell activated by an agonist. Our data demonstrate a close correlation between the ability of a ligand to promote the translocation of protein kinase C and its ability to phosphorylate endogenous substrates (Figures 1, 2, 4, and 5). This also seems to correlate well with the final biological response induced by the ligands involved (Nestor et al., 1982–1984). Under the experimental conditions described here, several phosphoproteins were revealed: 42-, 34-, 11-, and 10-kDa proteins and a 15-kDa protein that became partially dephosphorylated. At present, the identity of these proteins is not known, although several substrates for protein kinase C with similar apparent molecular weight have been described. Thus, a 40-kDa protein in human platelets has been identified with contradictory results by two laboratories either as lipocortin (Touqui et al, 1986) or as 1,4,5-triphosphate 5′-phosphomonoesterase (Connolly et al., 1986). A 37-kDa chromaffin granule membrane protein (Summers & Creutz, 1985) and a 36-kDa granule-associated protein from pituitary tissue (Turgeon & Copper, 1986) were identified as substrates for protein kinase C in cell-free systems. Whether some of the proteins described here are similar to these is not clear at the present time, nor is their role in the GnRH-mediated LH secretion.

In summary, we show that the potency and character (agonist, partial agonist, antagonist) of a GnRH analogue is reflected in its ability to induce (a) translocation of protein kinase C and (b) phosphorylation of endogenous substrates by the translocated (activated) enzyme. We characterize for the first time the substrates for the pituitary protein kinase C in vivo and propose an amplification cascade activated by a superagonist at the GnRH receptor level. These data butress the notion that protein kinase C is a mediator of the GnRH effects in the pituitary cell.

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Registry No. GnRH, 9034-40-6; [D-Nal(2)^6]GnRH, 76932-56-4; [Gly^2]GnRH, 37783-60-1; protein kinase, 9026-43-1.

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