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Characterization of pituitary calcium-activated, phospholipid-dependent protein kinase: Redistribution by gonadotropin-releasing hormone

(protein kinase C/phosphoinositide turnover/diacylglycerol)

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ABSTRACT **We report the presence in the rat pituitary of a calcium-activated, phospholipid-dependent protein kinase (C kinase), originally described by Takai *et al.* [Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979) *J. Biol. Chem.* 254, 3692–3695]. Enzyme activity is absolutely dependent on the simultaneous presence of Ca^{2+} and phospholipid—in particular, phosphatidylserine. The presence of small amounts of unsaturated diacylglycerol greatly increases the apparent affinity of the enzyme for Ca^{2+} and phosphatidylserine. Pituitary C kinase is mostly soluble (70%) and partly particulate (30%). Although the soluble form of the enzyme can be detected in a crude cytosol preparation, the particulate form is detectable only after solubilization and anion-exchange chromatography. Administration of a gonadotropin-releasing hormone (Gn-RH) agonist analog, [D-Ser(Bu^t)^6]des-Gly^{10}-Gn-RH-*N*-ethylamide, to ovariectomized rats resulted in elevated serum luteinizing hormone levels (245%) accompanied by a decrease in the cytosolic form of the enzyme (60%) and an increase in the particulate form (300%) after 5 min. This apparent activation of the particulate form seems to result from translocation of a soluble C kinase to the membrane. Several endogenous substrate proteins for C kinase ranging from 16 to 100 kDa were identified in pituitary cytosol. Pituitary C kinase might be involved in signal-transduction mechanisms in Gn-RH action, in particular, and in other hypophysiotropic hormones, in general, which operate by means of stimulation of phosphoinositide turnover during which diacylglycerol is liberated.**

The hypophysiotropic hormones of the hypothalamus exert their stimulatory effect on the pituitary either by cAMP production or by activating phosphoinositide turnover and Ca^{2+} mobilization (1–11). The newly discovered Ca^{2+}-activated, phospholipid-dependent protein kinase (C kinase) is activated by association with membrane phospholipids—in particular, phosphatidylserine (PtdSer)—in the presence of elevated Ca^{2+} (12). However, unsaturated diacylglycerol ($acyl_2Gro$) increases the apparent affinity for PtdSer and decreases the Ca^{2+} concentration needed for maximal enzyme activity (13). Since $acyl_2Gro$ is generated during phosphoinositide turnover, C kinase might be involved in mediating the action of hormones, neurotransmitters, growth factors, certain oncogene products, and other ligands that stimulate phosphoinositide turnover. Both gonadotropin-releasing hormone (Gn-RH) and thyrotropin-releasing hormone induce rapid phosphatidylinositol (PtdIns) turnover in the pituitary (4–11), and a preliminary study appeared describing pituitary C kinase (14). Therefore, the present study was undertaken to characterize the enzyme in the pituitary and to examine the effect of Gn-RH on C kinase activity.

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MATERIALS AND METHODS

Materials. PtdSer (bovine brain), cAMP, lysine-rich calf thymus histone (type III-s), phorbol 12-myristate 13-acetate (PMA), and diolein ($acyl_2Gro$) were from Sigma. [γ-^{32}P]ATP (>5000 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. [D-Ser(Bu^t)^6]des-Gly^{10}-Gn-RH-*N*-ethylamide was kindly provided by J. Sandow (Hoechst, A. G., F.R.G.).

***In Vivo* Experiments.** Wistar-derived female rats from the departmental colony were ovariectomized at 2 months of age and sacrificed about 1 month later. We took advantage of the fact that cells derived from pituitaries of ovariectomized rats (≈40 days after ovariectomy) contain about 23% of gonadotrophs as compared to 12% of gonadotrophs [luteinizing hormone (LH) + follicle-stimulating hormone cells] in a pituitary of adult female rat (G. V. Childs, personal communication). The Gn-RH superagonist was dissolved in saline (1 μg/0.2 ml) and injected into the caudal vein under ether anesthetic. Control rats received 0.2 ml of saline under similar conditions.

Enzyme Preparation. Pituitaries were collected and homogenized in ice-cold 20 mM Tris·HCl, pH 7.5/2 mM EDTA/50 mM 2-mercaptoethanol (buffer A). The homogenate was centrifuged at 4°C for 10 min at 100,000 × *g* [Beckman Airfuge, 30 psi (1 psi = 6.89 kPa)], and the supernatant solution was collected and used as the source of the crude soluble enzyme. The pellet was washed in buffer A, incubated with Triton X-100 (0.3%) for 1 hr at 4°C, and centrifuged for 10 min at 100,000 × *g*, and the supernatant was collected and used as the source of the crude particulate enzyme. For further purification, pituitaries from ovariectomized rats were collected in ice and homogenized in buffer A including 20% sucrose (buffer B). The homogenate was first centrifuged at 650 × *g* for 10 min and the supernatant was collected and further centrifuged for 10 min at 100,000 × *g* (Beckman Airfuge, 30 psi). The pellet was treated as described above and the extracts (soluble and particulate) were further fractionated on a DEAE-cellulose (DE-52) column (8.5 × 3 cm) equilibrated with buffer B. The column was washed with 15 ml of buffer B and protein kinases were eluted with a 30-ml linear concentration gradient of NaCl (0–0.4 M) in buffer B. Fractions (1 ml) were collected and assayed for protein kinase activity under various assay conditions. For comparative studies crude soluble and particulate enzyme were prepared from cerebral cortex and ovaries as described

Abbreviations: Gn-RH, gonadotropin-releasing hormone; C kinase, calcium-activated, phospholipid-dependent protein kinase; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; $acyl_2Gro$, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; LH, luteinizing hormone; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; A kinase, cAMP-dependent protein kinase; M kinase, proteolytic product of C kinase.

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above for the pituitary enzyme. Protein was determined by the Bradford method (15).

Enzyme Assay. C kinase was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into calf thymus histone (type III-s). The reaction mixture (0.150 ml) contained 5 μmol of Tris·HCl (pH 7.5), 1.25 μmol of $MgCl_2$, 50 μg of histone, 2.5 nmol of [γ-^{32}P]ATP (5–10 × 10^4 cpm/nmol), 50 μg of PtdSer per ml, 3.2 μg of diolein ($acyl_2$Gro) per ml, $CaCl_2$ (0.01 mM, unless otherwise indicated), and enzyme preparation as indicated. PtdSer and $acyl_2$Gro were first added in chloroform and dried under nitrogen. The residue was suspended in 20 mM Tris·HCl (pH 7.5) by sonication for 10 min at 4°C. The reaction was carried out for 3 min at 30°C and samples of 100 μl were removed and transferred to 2.5-cm squares of phosphocellulose paper. The papers were washed five times in a large volume of ice-cold trichloroacetic acid (10%), followed by a wash in ethanol and ether. The papers were then dried and the radioactivity was measured by liquid scintillation spectrometry. cAMP and Ca^{2+}-dependent protein kinase activities were determined similarly except that 0.5 μM cAMP or 1 mM Ca^{2+} was added and PtdSer and $acyl_2$Gro were omitted.

Protein Phosphorylation. Phosphorylation of endogenous cytosolic proteins was carried out similarly to the assay described above for C kinase, but in the absence of added histone. The assay was terminated by the addition of 70 μl of sample buffer (20 mM Tris·HCl, pH 7.5/15% mercaptoethanol/9% $NaDodSO_4$/30% glycerol/0.05% bromophenol blue) followed by placing the samples in boiling water for 3 min. $NaDodSO_4$/polyacrylamide electrophoresis of the phosphorylated samples (50 μl per lane; ≈50 μg of protein) was carried out by using 10% polyacrylamide gels, essentially as described by Laemmli (16). The gels were silver stained, dried under vacuum, and exposed for autoradiography (Curix RP2, AGFA) for 1–3 days.

Radioimmunoassay. Serum LH levels were determined by radioimmunoassay using the reagents provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Hormone Distribution Program, and are expressed in terms of the RP-1 rat LH reference.

RESULTS

C kinase activity was assayed in crude soluble and particulate pituitary preparations and in similar preparations derived from rat cerebral cortex and ovary.† C kinase activity in the cortex is mostly soluble (80%) and partially particulate (20%). In contrast, the majority of ovarian C kinase activity (60%) was recovered from the particulate fraction. Initial studies on crude pituitary preparations indicated that practically all of the enzyme was soluble. To further analyze the distribution of the enzyme in the pituitary, crude soluble and solubilized particulate preparations were fractionated by DEAE-cellulose chromatography (Fig. 1). A major peak of activity for both the soluble and particulate enzyme was eluted at about 0.16 M NaCl. The soluble and particulate forms of pituitary C kinase constitute 70% and 30% of the total enzyme activity, respectively. Most of the cAMP-dependent protein kinase (A kinase) eluted after C kinase, coinciding with a peak of kinase activity that is not dependent on the presence of Ca^{2+}, PtdSer, $acyl_2$Gro, or cAMP and can be detected even in the presence of 0.5 mM EGTA and 50 mM $MgCl_2$ (Fig. 1 and data not shown). It is very likely that this peak of activity represents M kinase, which is the proteolytic product of C kinase (17–20). In support of this assumption are the following observations, which are characteristic of M kinase (17–20): (*i*) M kinase was active even in the presence of 0.5 mM EGTA in the absence of Ca^{2+}, PtdSer, $acyl_2$Gro, or cAMP; (*ii*) its optimal Mg^{2+} concentration was 50 mM, whereas that of C kinase was 5 mM (Table 1); (*iii*) its preferred substrate was histone H2B, similar to A kinase but different from C kinase, which phosphorylates H1 histone better (Table 1); (*iv*) a similar elution profile of C and M kinases was observed recently in the adrenal gland (20). For reasons of simplicity, this peak of activity will be referred to as "M kinase" although further studies are needed to prove that this is indeed the catalytic domain of pituitary C kinase.

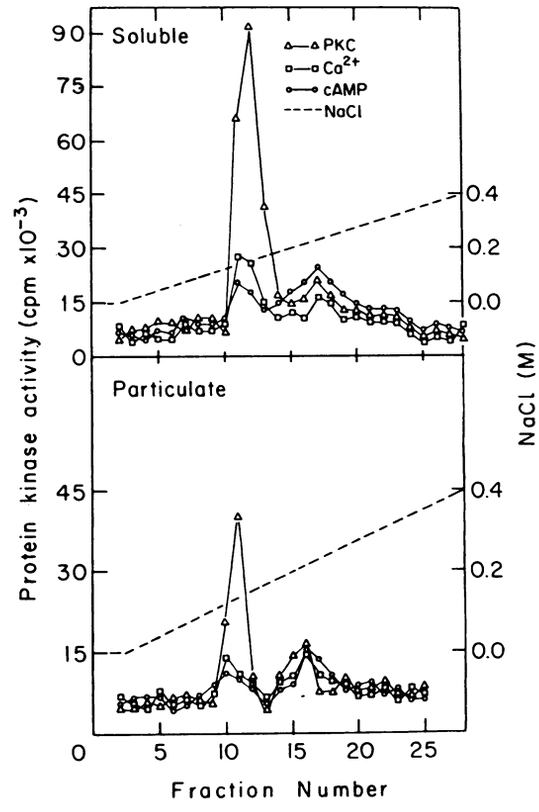

FIG. 1. DEAE-cellulose (DE-52) chromatography of pituitary C kinase. The 100,000 × *g* supernatant and particulate fractions were prepared from 45 anterior pituitaries. The column was first washed with 15 ml of buffer B and protein kinases were eluted with a 30-ml linear gradient of NaCl (0–0.4 M). Fractions of 1 ml were collected and 50-μl samples were assayed for cAMP-dependent protein kinase (A kinase, 0.5 μM cAMP), for Ca^{2+}-dependent protein kinase (1 mM Ca^{2+}), and for C kinase (PKC) (1 mM Ca^{2+}, PtdSer at 50 μg/ml, and $acyl_2$Gro at 3.2 μg/ml).

Table 1. Some properties of the various kinases in the pituitary

Protein	Relative protein kinase activity, %		Optimal Mg^{2+}, mM
	Histone H1	Histone H2B	
C kinase	100	58	5
A kinase	50	100	ND
M kinase	84	100	50

Crude soluble enzyme preparation was fractionated on a DE-52 column as described in the legend to Fig. 1. Substrate specificities are compared for histones H1 and H2B. The respective peaks were assayed for C kinase (1 mM Ca^{2+}, PtdSer at 50 μg/ml, and $acyl_2$Gro at 3.2 μg/ml), for A kinase (0.5 μM cAMP), and for M kinase (25 mM Mg^{2+} and 0.5 mM EGTA). ND, not determined.

†Twenty-eight-day-old female rats treated with 15 international units of pregnant mare serum gonadotropin on day 26 to induce follicular growth.

In six different experiments "M kinase" eluted after C kinase, as also observed in bovine adrenocortical tissue (20).

The contribution of the various cofactors needed for enzyme activity is shown in Fig. 2. Calcium, acyl$_2$Gro, PtdSer, and the potent tumor promoter phorbol ester PMA were weak stimulants of C kinase activity when added alone. Likewise, the combination of Ca^{2+} + acyl$_2$Gro or Ca^{2+} + PMA was not sufficient to approach maximal enzyme activity. However, the simutaneous presence of Ca^{2+} and PtdSer markedly increased C kinase activity. The presence of small amounts of acyl$_2$Gro or PMA further increased enzyme activity compared to Ca^{2+} + PtdSer alone. Analysis of the stimulatory effect exerted by acyl$_2$Gro in crude cytosol preparations revealed that in its presence the K_a for Ca^{2+} and PtdSer decreased from 32 μM and 30 μg/ml to 10 μM and 15 μg/ml, respectively. In a partially purified enzyme preparation the presence of acyl$_2$Gro decreased the K_a for Ca^{2+} from 4 μM to 1.6 μM. When acyl$_2$Gro or PMA was present in the reaction mixture with PtdSer, significant enhanced activity of C kinase was detected even at 0.1 μM Ca^{2+} (not shown).

PtdSer is the preferred phospholipid for pituitary C kinase activation (Table 2), whereas the other major phospholipids could only support 20–35% of maximal enzyme activity, the order being PtdSer > PtdIns > phosphatidylethanolamine (PtdEtn) > phosphatidylcholine (PtdCho).

Administration of [D-Ser(Bu^t)^6]des-Gly^{10}-Gn-RH-*N*-ethylamide (1 μg per rat) to ovariectomized rats resulted in elevated serum LH levels {control, 1.8 ± 0.35 μg/ml; [D-Ser(Bu^t)^6]des-Gly^{10}-Gn-RH-*N*-ethylamide, 4.4 ± 0.6 μg/ml} concomitantly with a decrease (60%) in soluble C kinase activity and an increase (300%) in the particulate form activity after 5 min (Fig. 3). Data pooled from seven experiments revealed an average decrease of 50% in soluble pituitary C kinase activity and an increase of 270% in the particulate form after Gn-RH analog administration. [D-Ser-(Bu^t)^6]des-Gly^{10}-Gn-RH-*N*-ethylamide had no significant effect on "M kinase" activity.

Phosphorylation of cytosolic pituitary proteins revealed that in the presence of Ca^{2+}, PtdSer, and acyl$_2$Gro, several endogenous substrate proteins were markedly phosphorylated (Fig. 4). The presence of Ca^{2+}, PtdSer, or acyl$_2$Gro alone had only a small effect on the phosphorylation of the substrate proteins (Fig. 4 and data not shown). Several endogenous substrate proteins for pituitary C kinase were identified: 16, 18, 19, 21, 23, 28, 35, 40, 50, 71, 78, 92, and 100 kDa (Fig. 4, and data from several other gels).

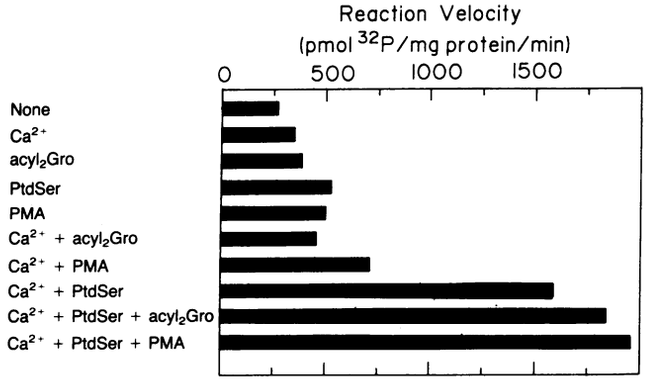

FIG. 2. Contribution of Ca^{2+}, diolein (acyl$_2$Gro), PtdSer, and phorbol ester (PMA) to C kinase activity. Crude soluble enzyme preparation was fractionated on a DE-52 column as described in the legend to Fig. 1 and the respective peak of C kinase was used for the assay. None, 0.5 mM EGTA; Ca^{2+}, 10 μM; acyl$_2$Gro, 3.2 μg/ml; PtdSer, 50 μg/ml; PMA, 20 ng/ml.

Table 2. Relative efficiency of phospholipids for activation of pituitary C kinase

Treatment	C kinase activity, pmol of ^{32}P per mg of protein per min	% activity
Ca^{2+}	497	—
+ PtdSer	4444	100
+ PtdIns	1895	35.4
+ PtdEtn	1588	27.6
+ PtdCho	1267	19.5

Crude soluble enzyme preparation was fractionated on a DE-52 column as described in the legend to Fig. 1. The respective peak of C kinase was used for the assay. The enzyme assay included Ca^{2+} alone or Ca^{2+} (10 μM), diolein (3.2 μg/ml), and the phospholipid indicated at 50 μg/ml. For percent activity the values obtained with Ca^{2+} alone were subtracted from the values obtained with Ca^{2+} + phospholipid.

DISCUSSION

Phosphorylation of endogenous cellular proteins following hormone–receptor activation is a key step in signal transduction mechanisms (21). Peptide hormones, neurotransmitters, and growth factors induce covalent modification of endogenous proteins by the formation of a second messenger. In addition to the well-known role of cyclic nucleotides and Ca^{2+} as second messengers, acyl$_2$Gro has drawn attention recently to its potential role in signal transduction. Released during phosphoinositide turnover, acyl$_2$Gro can activate C kinase in the face of micromolar Ca^{2+} concentrations and the presence of PtdSer (18, 19). Therefore, pituitary C kinase is a potential mediator for hypophysiotropic hormones that act by means of increased PtdIns turnover (e.g., thyrotropin-releasing hormone and Gn-RH; refs. 4–11).

The present report demonstrates the presence of C kinase in the pituitary and modulation of its activity by Gn-RH. The enzyme is mostly soluble (70%) and partly particulate (30%). However, although the particulate C kinase of the ovary or cerebral cortex could be demonstrated even in a crude enzyme preparation, the pituitary enzyme was detected only after solubilization and anion-exchange chromatography. The presence of protease inhibitors such as leupeptin or phenylmethylsulfonyl fluoride during enzyme preparation and assay was not sufficient to demonstrate enzymatic activity in the crude particulate fraction. Also the presence of Triton X-100 in the crude preparation is not responsible for the lack of measurable activity, since under similar conditions we found C kinase activity in cortex and ovarian particulate preparations. Moreover, addition of Triton X-100 to the soluble enzyme under similar conditions to those used for solubilization of the particulate enzyme had only a slight inhibitory effect on C kinase activity. Therefore, it is possible that the particulate fraction of the pituitary contains an inhibitor of C kinase that was removed during fractionation. Whether the inhibitory activity is specific to C kinase or represents a Ca^{2+}-dependent neutral protease such as the calpain family (22) is not clear at the present time. Similar subcellular distribution of C kinase is found in guinea pig or rat heart and cerebral cortex, whereas in spleen it is divided almost equally between the cytosolic and particulate fractions (23). In human neutrophils and leukemia cells, the enzyme is almost exclusively localized in the particulate form (23).

Pituitary C kinase has an absolute requirement for Ca^{2+} and phospholipid—in particular, PtdSer. Other phospholipids tested could only partially support enzyme activity, PtdSer > PtdIns > PtdEtn > PtdCho. The presence of a small amount of acyl$_2$Gro decreases the K_a for PtdSer and Ca^{2+}, lending support to the suggestion that increased phospho-

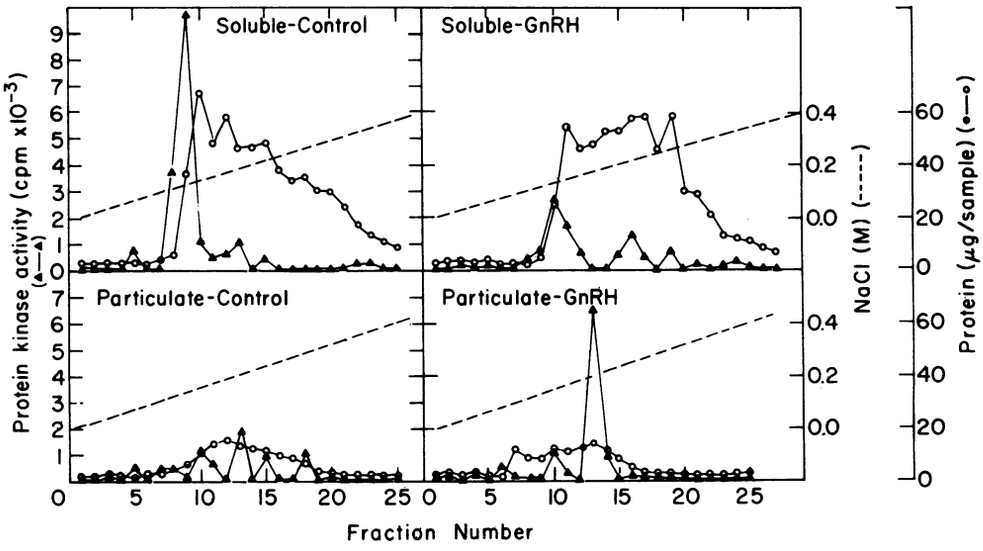

FIG. 3. Effect of Gn-RH on intracellular redistribution of C kinase. Gn-RH agonist [D-Ser(Bu^t)^6]des-Gly^{10}-Gn-RH-*N*-ethylamide was administered to 30 ovariectomized rats (i.v.). The control group received saline. After 5 min, rats were sacrificed and enzyme preparation and chromatography on DE-52 column were carried out as described in the legend to Fig. 1. Enzyme activity obtained with Ca^{2+} alone was subtracted from the values obtained with Ca^{2+} + phospholipid.

inositide turnover in the pituitary results in C kinase activation.

Our observations that Gn-RH treatment resulted in a decrease in soluble C kinase activity and increase in the particulate form suggest redistribution of the enzyme by Gn-RH. We could not rule out completely the possibility that the effect observed here by Gn-RH was due to separate effects on soluble and particulate enzyme activities. Nevertheless, the hypothesis that the major process was a translocation of a soluble enzyme to the membrane was strengthened by several observations: (*i*) most of the pituitary enzyme is soluble and inactive, and thus recruitment of the enzyme to the membrane is a means of activating it by exposure to $acyl_2Gro$ that is released during enhanced PtdIns turnover; (*ii*) the fact that no significant changes in the total C kinase activity after Gn-RH treatment were observed (Fig. 3 and data not shown); (*iii*) the reports that phorbol ester causes a similar translocation of C kinase in parietal yolk sac cells (24) and mimics the stimulatory effect of Gn-RH on pituitary LH release (25, 26); (*iv*) the observation that Gn-RH translocates calmodulin from the cytosol to the membrane (27). Translocation of C kinase to the membrane by Gn-RH following increased PtdIns turnover will expose the enzyme to $acyl_2Gro$ and PtdSer present in the membrane and enable activation even in the presence of low concentrations of Ca^{2+}. Although Gn-RH was administered *in vivo*, it is unlikely that the effect observed was not exerted at the gonadotroph level since we have demonstrated previously that Gn-RH binds and activates only the gonadotrophs (28). Nevertheless, attempts should be made to improve the yield of purified gonadotrophs in order to follow the direct effect of Gn-RH on C kinase in purified gonadotrophs. The recent preliminary findings that synthetic $acyl_2Gro$ mimics the effect of Gn-RH on LH release (29, 37) and that retinol inhibits pituitary C kinase activity and Gn-RH-induced LH release (38) provide further support of the proposal that pituitary C kinase is involved in Gn-RH-induced gonadotropin release.

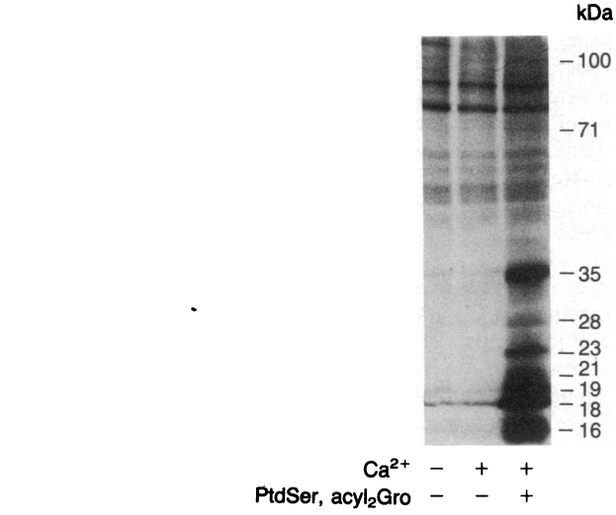

FIG. 4. Autoradiogram of endogenous substrate proteins for pituitary C kinase. Additions were made as indicated: 1 mM Ca^{2+}; PtdSer at 50 μg/ml; diolein ($acyl_2Gro$) at 3.2 μg/ml. Phosphorylation was carried out for 3 min at 30°C.

Interestingly, it was reported recently that corticotropin causes a shift of membrane-associated C kinase to a soluble form in adrenocortical cells (20). It is possible that since cAMP mediates many actions of corticotropin, the effect on C kinase might be a secondary feedback effect to oppose cAMP-induced actions. On the other hand, we suggest that in the pituitary C kinase is part of the "second messenger" cascade that accounts for the difference between the two systems.

The possibility that the decrease in cytosolic pituitary C kinase activity after Gn-RH treatment resulted from proteolytic cleavage of the enzyme and conversion to M kinase seems unlikely since Gn-RH treatment showed no stimulatory effect on "M kinase" activity.

The physiological substrates for C kinase are still not known but several endogenous proteins were reported recently to be phosphorylated by the enzyme—among them a 40-kDa protein in human platelets (30), the cytoskeletal protein vinculin (31), an 80-kDa protein in the 3T3 cell line (32), smooth muscle heavy meromyosin (33), myosin light chain (34), myelin basic proteins, troponins I and T, filamin, phosphorylase kinase, glycogen synthetase, and eukaryotic initiation factor 2 (for review, see refs. 23 and 35). Initial studies in our laboratory revealed several endogenous phosphoproteins that were labeled by C kinase in the pitu-

itary (Fig. 4), in good agreement with substrate proteins for rat cerebral cortex C kinase (36). Whether some of the proteins are similar to those described above and whether Gn-RH affects their phosphorylation *in vivo* is not clear at the present time.

Pituitary C kinase characterized here might serve a key regulatory role in signal transduction for Gn-RH-induced gonadotropin release, in particular, and for other hypophysiotropic hormones (e.g., thyrotropin-releasing hormone) that exert their biological action by enhanced phosphoinositide turnover.

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