Growth Hormone Secretion in Human Acromegalic Pituitary Adenomas: Cyclic Adenosine Monophosphate and Protein Kinase C Responses

Tatsuo Ishizuka, Hiroyuki Morita, Tomoatsu Mune, Hisashi Daidoh, Junko Hanafusa, Mayumi Yamamoto, Toshiroh Shibata, and Keigo Yasuda

We examined the effect of phorbol ester on growth hormone (GH)-releasing hormone (GRH)-induced GH secretion and cyclic adenosine monophosphate (cAMP) production in three pituitary adenomas and thyrotropin-releasing hormone (TRH)- and corticotropin-releasing hormone (CRH)-induced redistribution of protein kinase C (PKC) from cytosol to membrane in a pituitary adenoma resected from patients with acromegaly. GRH stimulated GH secretion in accordance with cAMP production in three cases, whereas 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) stimulated GH secretion with cAMP production in one case. Simultaneous addition of GRH and TPA enhanced cAMP production in three pituitary adenomas. Moreover, addition of TPA with GRH resulted in additive secretion of GH in vitro. In one case, we were able to measure PKC activity and prove translocation of PKC stimulated by TRH and CRH in accordance with GH secretion in vitro and in vivo. These results suggest that TPA, an activator of PKC, has a stimulatory effect on GRH-induced cAMP production and that, finally, TRH- and CRH-induced PKC activation may cause greater secretion of GH by enhancement of cAMP production in human GH-hypersecreting adenoma cells.

MODULATING the transduction efficiency of plasma membrane hormone receptors is important for an adaptive response to multiple incoming hormonal signals. Since many types of cell surface receptors can stimulate protein kinases, such as cyclic adenosine monophosphate (cAMP)-dependent protein kinase, calcium/phospholipid-dependent protein kinase, and calmodulin-dependent protein kinase, these kinases can affect homologous and/or heterologous hormone receptor-effector systems. Phorbol esters increase basal and hormone-stimulated cAMP accumulation in the pituitary[1-5] and in a number of other tissues.[6-9] In addition, several investigators have demonstrated that membrane preparations from cells exposed to phorbol esters exhibit increased basal and hormone-stimulated adenylate cyclase activity.[5,8,10] Thus, it appears that protein kinase C (PKC) activation can increase cAMP synthesis in some cell types. In most studies to date, PKC has been activated in intact cultured cells, and the effects on adenylate cyclase activity were characterized in prepared plasma membranes.

We determined that a stimulatory effect of PKC on cAMP production and thyrotropin-releasing hormone (TRH)- and corticotropin-releasing hormone (CRH)-induced PKC activation are involved in growth hormone (GH) secretion in human GH-secreting pituitary adenomas.

SUBJECTS AND METHODS

Patients

Pituitary tumors (four GH-secreting tumors) were collected in the operating room after transsphenoidal adenomectomy. Fragments of tumors were immediately incubated with Krebs-Ringer-phosphate buffer (KRP), pH 7.4, containing 1% bovine serum albumin, (BSA). Other fragments were used for light microscopy and immunocytochemical studies. Diagnosis before surgery was established on the basis of clinical and radiological criteria as indicated in Table 1.

Materials

Phosphatidylserine, diolein, histone (type III-S), phenylmethylsulfonyl fluoride (PMSF), leupeptin, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), BSA, and ATP were purchased from Sigma Chemical (Tokyo, Japan). [γ-^{32}P]ATP (3,000 Ci/mmol) and [^{32}P]orthophosphate (1.0 mCi/mL] were purchased from New England Nuclear (Tokyo, Japan). Each GH-releasing hormone (GRH) and CRH was purchased from Sumitomo Pharmaceutical (Osaka, Japan) and the Peptide Institute Protein Research Foundation (Osaka, Japan), respectively. All other chemicals were of reagent grade or better.

Isolated Adenoma Cell Experiments

Isolated pituitary adenoma cells were prepared by collagenase (Worthington Biochemical, Freehold, NJ) digestion of human resected pituitary adenoma tissue from three patients with acromegaly in KRP (pH 7.4) containing 127 mmol/L NaCl, 12.3 mmol/L NaH_2PO_4, 5.1 mmol/L KCl, 1.3 mmol/L $MgSO_4$, 1.4 mmol/L $CaCl_2$, 3% BSA, and 2.5 mmol/L glucose.[11] Adenoma cells were washed and preincubated at 37°C in glucose-free KRP containing 1% BSA for 30 minutes, and were then incubated with or without (control) 100 nmol/L GRH, 100 nmol/L CRH, 1 μmol/L TPA, 100 nmol/L GRH plus 1 μmol/L TPA, or 100 nmol/L CRH plus 1 μmol/L TPA for 0, 5, 15, 30, and 60 minutes. In the GH-secretion study, the reaction was terminated by addition of ice-cold glucose-free KRP containing 1% BSA. In the PKC study, the reaction was terminated by addition of 20 mmol/L Tris hydrochloride buffer (pH 7.5) containing 0.25 mol/L sucrose, 1.2 mmol/L EGTA, 0.1 mmol/L PMSF, 20 μg/mL leupeptin, and 20 mmol/L 2-mercaptoethanol (buffer I), and cells were washed twice and homogenized in buffer I. The homogenates were centrifuged for 60 minutes at 105,000 × *g* to obtain cytosol and membrane fractions. The latter was homogenized in buffer I containing 5 mmol/L EGTA, 2 mmol/L EDTA, and 1% Triton X-100.

PKC Studies

To measure PKC activity of pituitary adenoma cells, the cytosol or solubilized-membrane fraction was applied onto a DEAE

From the Third Department of Internal Medicine, Gifu University School of Medicine, Gifu, Japan.

Submitted February 27, 1995; accepted June 24, 1995.

Address reprint requests to Tatsuo Ishizuka, MD, Third Department of Internal Medicine, Gifu University School of Medicine, Tsukasa-machi 40, Gifu 500, Japan.

0026-0495/96/4502-0013$03.00/0

Table 1. Clinical Characteristics and Various Agonist-Induced GH Responses in Four Patients With GH-Secreting Adenomas

Patient No.	Sex/ Age (yr)	GH Level (ng/mL)	After Stimulation With Agonist (min) 0	15	30	60	90	120	Adenoma Size (cm)	Histology
1	F/45	GRH	134	704	863	588	452	319	3.0 × 3.2 × 2.9	Acidophil
		CRH	178	392	500	229	141	138		
		TRH	166		656	321	138	152		
		LHRH	125		314	209	175	162		
		Bromo	289		210	201	198	192		
2	M/34	TRH	41		96	81		72	2.3 × 1.8 × 1.6	Chromophobe
		Bromo	80		83	76	69	64		
3	F/65	GRH	24	128	136	112	75	58	2.0 × 1.7 × 1.5	Acidophil
		CRH	24	14	12	9	12	17		
		TRH	22	32	34	26	22	22		
		LHRH	20	21	23	22	18	17		
		Bromo	20	24	14	12	9	12		
		SMS	18		14	5	7	6		
4	M/33	GRH	45	215	266	193	117	104	2.0 × 2.8 × 1.0	Acidophil
		CRH	53	68	72	73	60	59		
		TRH	62	81	76	69	72	66		
		LHRH	61	161	239	172		86		
		Bromo	111		82	70	79	81		
		SMS	60			26		36		

Abbreviations: GRH, 100 μg GRH intravenous injection; TRH, 500 μg TRH intravenous injection; CRH, 100 μg CRH intravenous injection; Bromo, 2.5 mg bromocriptine oral administration; SMS, 100 μg octreotide acetate subcutaneous injection.

Sephacel (Pharmacia Biotech, Tokyo, Japan) column (bed volume, 200 μL) that had been equilibrated with buffer II (20 mmol/L Tris hydrochloride, pH 7.5, 2 mmol/L EGTA, 20 mmol/L 2-mercaptoethanol, 20 μg/mL leupeptin, and 0.1 mmol/L PMSF) and eluted with 600 μL 0.1-mol/L NaCl in buffer II.[12] The eluted fraction was concentrated by Centricon tube (Amikon; Rikaken, Tokyo, Japan) and used for PKC assay. PKC activity was assayed by measuring phosphorylation of histone (type III-S) as described previously.[12]

Measurement of GH Secretion and cAMP Content of Adenoma Cells

After cell suspensions were incubated with agonists, reactions were terminated by addition of ice-cold KRP as specified in the figures. Then suspensions were centrifuged at 2,000 rpm for 5 minutes. The resultant supernatant was subjected to radioimmunoassay of GH (Growth Hormone RIA Kit; Dinabot, Tokyo, Japan). The pellet was homogenized in 150 μL 0.1N HCl and subjected to radioimmunoassay (Cyclic AMP Kit "Yamasa"; Yamasa, Tokyo, Japan).

RESULTS

GRH-, CRH-, TPA, or GRH Plus TPA–Induced GH Secretion in Human Pituitary Adenoma Cells

Clinical characteristics and TRH-, CRH-, or luteinizing hormone–releasing hormone (LHRH)-induced GH secretion in vivo before operation in case no. 1 and no. 4 are indicated in Table 1. TRH- and CRH-induced in vitro GH secretion was also observed in isolated human pituitary adenoma cells (Fig 3). When incubated with 100 nmol/L GRH for 60 minutes, GH secretion in adenoma cells was increased in case no. 2. However, neither 100-nmol/L CRH-induced GH secretion nor 1-μmol/L TPA-induced secretion was observed. Simultaneous addition of TPA and GRH enhanced GRH-induced GH secretion in pituitary adenoma cells (Fig 1a). GRH plus TPA–induced GH secretion was also maximal in the other two cases (Fig 1b and c). In case no. 4, TPA-induced GH secretion was slightly increased (120% for 60 minutes during stimulation with TPA) in accordance with cAMP production (Figs 1c and 2c).

GRH-, TPA-, or GRH Plus TPA–Induced cAMP Production in Human Pituitary Adenoma Cells

In case no. 2, 100 nmol/L GRH induced a 13-fold increase of cAMP production for 15 minutes, as indicated in Fig 2a. Simultaneous addition of GRH and TPA resulted in a 23-fold increase of cAMP production. Accordingly, TPA enhanced GRH-induced cAMP production. In cases no. 3 and 4 (Fig 2b and c), as well as case no. 2, simultaneous addition of GRH and TPA also enhanced GRH-induced cAMP production. In three cases, TPA alone stimulated cAMP production (twofold to 60-fold increase).

Redistribution of PKC in Pituitary Adenoma Cells

When incubated with 100 nmol/L TRH, cytosolic PKC activity decreased and membrane-associated PKC activity increased symmetrically for 0.25 minutes in case no. 1 (Fig 3A). During stimulation with 10 nmol/L CRH for 3 minutes, cytosolic activity gradually decreased and membrane-associated activity increased for 5 minutes (Fig 3B). In other cases, PKC activity was not measured because of paucity of the sample.

DISCUSSION

In this study, we have documented TPA-enhanced GRH-induced cAMP production and TRH-induced redistribution of PKC from cytosol to membrane in GH-secreting pituitary adenoma cells.

It has been reported that TRH induced translocation of PKC in rat pituitary cells and cultured GH_3 and GH_4C_1

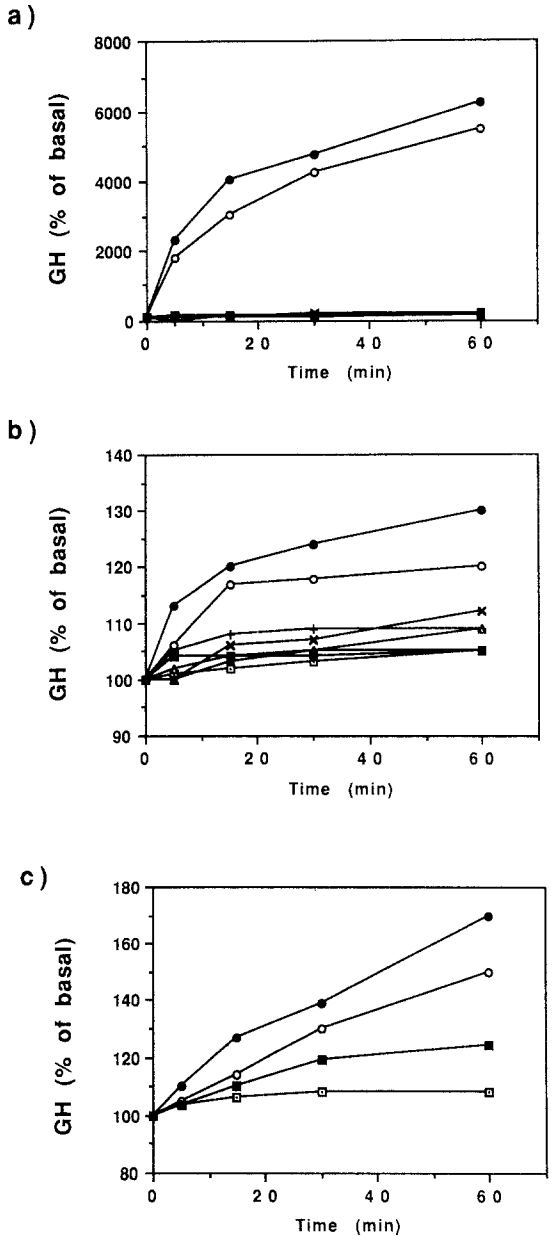

Fig 1. GH secretion by 100 nmol/L GRH (○), 100 nmol/L CRH (†), 1 μmol/L TPA (■), 100 nmol/L CRH plus 1 μmol/L TPA (×), 100 nmol/L TRH plus 1 μmol/L TPA (▲), or 100 nmol/L GRH plus 1 μmol/L TPA (●) in human pituitary adenoma cells. (a) Case no. 2; (b) case no. 3; (c) case no. 4. Cell suspensions (10^4 cells per tube) were preincubated for 15 minutes, stimulated with or without (control, □) various agonists for 0, 5, 15, 30, and 60 minutes, and terminated with ice-cold KRP. Values are the mean of duplicate determinations.

cells.[13-17] On the other hand, human GRH[18-20] or vasoactive intestinal peptide[21] stimulate intracellular cAMP accumulation, leading to GH secretion. Previously, GRH-stimulated cAMP production has been shown to be amplified by direct PKC activation.[2,22,23] In fact, we have demonstrated here that in human GH-hypersecreting pituitary adenoma cells, phorbol ester stimulates GRH-induced cAMP production, and the enhanced cAMP production seems to potentiate GH secretion. The reason that GH secretion by GRH plus TPA did not parallel cAMP production in our cases may be as follows. First, stimulation with 100 nmol/L GRH is probably maximal; therefore, even if cAMP production is increased by addition of TPA with GRH, GH secretion by GRH plus TPA apparently does not overcome the GRH-induced secretion. In fact, it has already been reported that TPA added with GRH induces an additive GH-secretory response.[24,25] Therefore, it is suggested that simultaneous

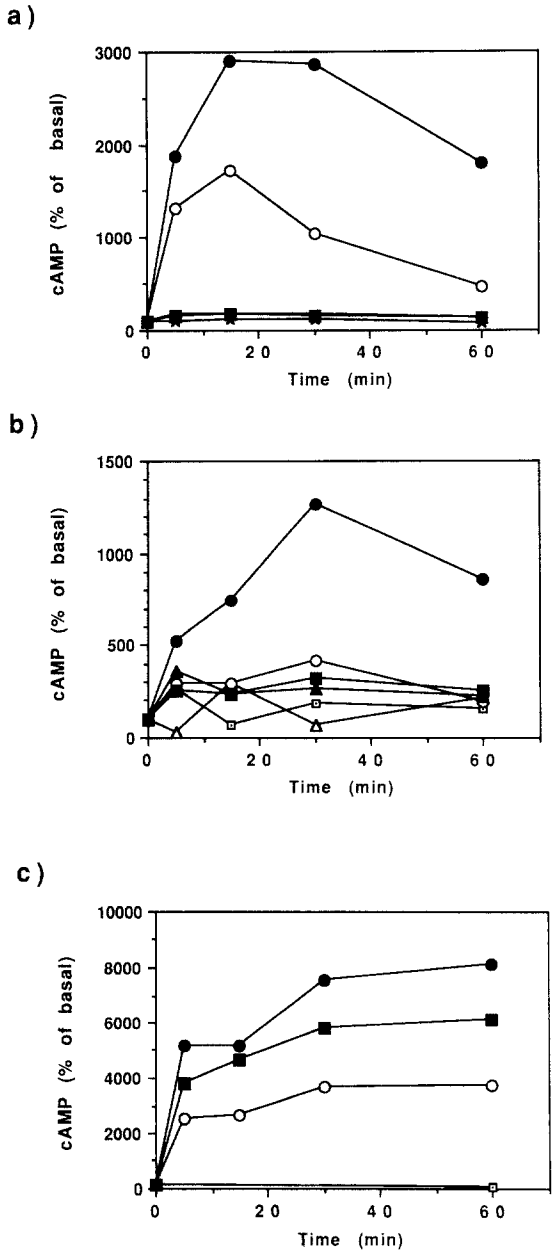

Fig 2. (○) 100 nmol/L GRH-, (△) 100 nmol/L TRH-, (■) 1 μmol/L TPA-, (×) 100 nmol/L CRH plus 1 μmol/L TPA–, (▲) 100 nmol/L TRH plus 1 μmol/L TPA–, or (●) 100 nmol/L GRH plus 1 μmol/L TPA–induced cAMP production in human pituitary adenoma cells. (a) Case no. 2; (b) case no. 3; (c) case no. 4. Cell suspensions (10^4 cells per tube) were preincubated for 15 minutes, stimulated with or without (control, □) various agonists for 0, 5, 30, and 60 minutes, and terminated with ice-cold KRP. Values are the mean of duplicate determinations.

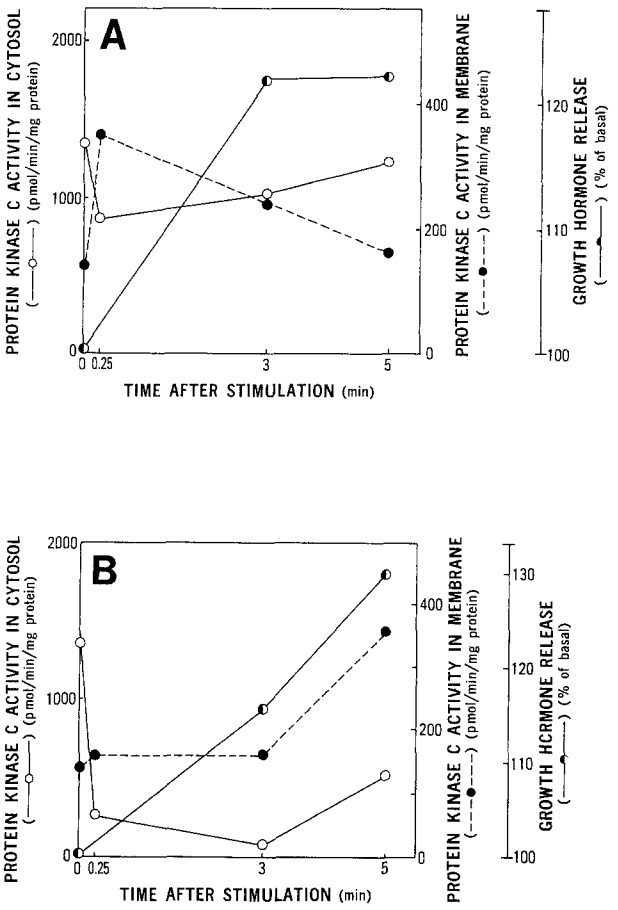

Fig 3. TRH- and CRH-induced redistribution of PKC activity and GH secretion in human pituitary adenoma cells (case no. 1). Cell suspensions (10^5 to 10^6 cells per tube) were incubated with 100 nmol/L TRH (A) or 10 nmol/L CRH (B) for 0, 0.25, 3, and 5 minutes after preincubation for 15 minutes. Values are the mean of duplicate determinations.

addition of a low concentration of GRH with phorbol ester may result in some additive effect of phorbol ester on GRH-induced GH secretion. Second, it is possible that an increase of cAMP production regulates PIP_2 hydrolysis and phorbol ester–induced GH secretion, as reported in other cell types.[26-28] Third, altered stimulatory guanine nucleotide trisphosphate (GTP)-binding protein (Gs) may result in continuous stimulation of adenylate cyclase activity and GH secretion, and accordingly, pituitary adenoma cells responded poorly to the stimulatory agents, as reported previously.[29,30] This is because adenylate cyclase regulates GTPase-activating protein activity and effectively turns off the response.[31]

However, the mechanism by which phorbol ester enhances GRH-induced cAMP production is still unclear. PKC-mediated inhibition of phosphodiesterase and enhancement of adenylate cyclase activity in rat anterior pituitary cells were suggested as explanations for phorbol ester–enhanced CRH-induced cAMP production.[4] Moreover, Bell et al[8] observed a putative stimulatory effect of PKC on α_s-GTP-catalytic subunit interaction in S49 lymphoma cells. More recently, some investigators have reported point mutations of Gsα and PKC in human pituitary adenomas.[30,32] More experiments will be required to elucidate the hypersecretion of GH in human pituitary adenomas.

In summary, we have demonstrated that in human pituitary GH-secreting pituitary adenomas resected from four patients with acromegaly, (1) TRH and CRH induced redistribution of PKC from cytosol to membrane in the case of CRH-induced paradoxical GH secretion, and (2) TPA enhanced GRH-induced cAMP production. These results suggest that dual mechanisms for GH hypersecretion, namely the PKC activation system and the cAMP synthesis system, are synergistic in human pituitary GH-secreting adenomas.

REFERENCES

1. Cronin MJ, Canonico PL: Tumor promoters enhance basal and growth hormone releasing factor stimulated cyclic AMP levels in anterior pituitary cells. Biochem Biophys Res Commun 129:404-410, 1985

2. Cronin MJ, Summers ST, Sortino MA, et al: Protein kinase C enhances growth hormone releasing factor (1-40)–stimulated cyclic AMP levels in anterior pituitary. Actions of somatostatin and pertussis toxin. J Biol Chem 261:13932-13935, 1986

3. Quilliam LA, Dobson PRM, Brown BL: Modulation of cyclic AMP accumulation in GH3 cells by a phorbol ester and thyroliberin. Biochem Biophys Res Commun 129:898-903, 1985

4. Abou-Samura A, Harwood JP, Manganiello VC, et al: Phorbol 12-myristate 13-acetate and vasopressin potentiate the effect of corticotropin-releasing factor on cyclic AMP production in rat pituitary cells. J Biol Chem 262:1129-1136, 1987

5. Summers ST, Cronin MJ: Phorbol esters enhance basal and stimulated adenylate cyclase activity in a pituitary cell line. Biochem Biophys Res Commun 135:276-281, 1986

6. Sugden D, Vanecek J, Klein DC, et al: Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature 314:359-361, 1985

7. Simantov R, Sachs L: Enhancement of hormone action by a phorbol ester and anti-tubulin alkaloids involves different mechanisms. Biochim Biophys Acta 720:120-125, 1982

8. Bell J, Buxton ILO, Brunton LL: Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters. Putative effect of C kinase on α_s-GTP-catalytic subunit interaction. J Biol Chem 260:2625-2628, 1985

9. Nabika T, Nara Y, Yamori Y, et al: Angiotensin II and phorbol ester enhance isoproterenol- and vasoactive intestinal peptide (VIP)-induced cyclic AMP accumulation in vascular smooth muscle cells. Biochem Biophys Res Commun 131:30-36, 1985

10. Jakobs KH, Bauer S, Watanabe Y: Modulation of adenylate cyclase of human platelets by phorbol ester. Impairment of the hormone-sensitive inhibitory pathway. Eur J Biochem 151:425-430, 1985

11. Ishizuka T, Ito Y, Murayama M, et al: Hormone-induced redistribution of protein kinase C in human pituitary adenomas. Clin Chim Acta 170:351-354, 1987

12. Ishizuka T, Miura K, Nagao S, et al: Differential redistribution of protein kinase C in human aldosteronoma cells and adjacent adrenal cells stimulated with ACTH and angiotensin II. Biochem Biophys Res Commun 155:643-649, 1988

13. Zatz M, Mahan LC, Reisine T: Translocation of protein

kinase C in anterior pituitary tumor cells. J Neurochem 48:106-110, 1987

14. Drust DS, Martin TFJ: Protein kinase C translocates from cytosol to membrane upon hormone activation: Effect of thyrotropin-releasing hormone in GH_3 cells. Biochem Biophys Res Commun 128:531-537, 1985

15. Turgeon JL, Cooper RH: Protein kinase C and an endogenous substrate associated with adenohypophyseal secretory granules. Biochem J 237:53-61, 1986

16. Martinez de la Escalera G, Porter BW, Martin TFJ, et al: Dopamine withdrawal and addition of thyrotropin-releasing hormone stimulate membrane translocation of protein kinase-C and phosphorylation of an endogenous 80 K substrate in enriched lactotrophs. Endocrinology 25:1168-1173, 1989

17. Feron CW, Tashjian AH Jr: Thyrotropin-releasing hormone induces redistribution of protein kinase C in GH_4C_1 rat pituitary cells. J Biol Chem 260:8366-8371, 1985

18. Bilezikjian LM, Vale WW: Stimulation of adenosine 3′,5′-monophosphate production by growth hormone–releasing factor and its inhibition by somatostatin in anterior pituitary cells in vitro. Endocrinology 113:1726-1731, 1983

19. Cronin MJ, Hewlett EL, Evans WS, et al: Human pancreatic tumor growth hormone (GH)-releasing factor and cyclic adenosine 3′,5′-monophosphate evoke GH release from anterior pituitary cells: The effects of pertussis toxin, cholera toxin, forskolin, and cycloheximide. Endocrinology 114:904-913, 1984

20. Spada A, Vallar L, Giannattasio G: Presence of an adenylate cyclase dually regulated by somatostatin and human pancreatic growth hormone (GH)-releasing factor in GH-secreting cells. Endocrinology 115:1203-1209, 1984

21. Dorflinger LJ, Schonbrunn A: Somatostatin inhibits vasoactive intestinal peptide–stimulated cyclic adenosine monophosphate accumulation in GH pituitary cells. Endocrinology 113:1541-1551, 1983

22. Summers ST, Cronin MJ: Phorbol esters enhance basal and stimulated adenylate cyclase activity in a pituitary cell line. Biochem Biophys Res Commun 135:276-281, 1986

23. Summers ST, Walker JM, Sando JJ, et al: Phorbol esters increase adenylate cyclase activity and stability in pituitary membranes. Biochem Biophys Res Commun 151:16-24, 1988

24. Ohmura E, Friesen HG: 12-*O*-Tetradecanoyl phorbol-13-acetate stimulates rat growth hormone (GH) release through different pathways from that of human pancreatic GH-releasing factor. Endocrinology 116:728-733, 1985

25. Summers ST, Canonico PL, MacLeod RL, et al: Phorbol esters affect pituitary growth hormone (GH) and prolactin release: The interaction with GH releasing factor, somatostatin and bromocriptine. Eur J Pharmacol 111:371-376, 1985

26. Nishizuka Y: Intracellular signaling by hydrolysis of phospholipid and activation of protein kinase C. Science 233:305-312, 1986

27. Takai Y, Kaibuchi K, Nishizuka Y: Counteraction of calcium-activated, phospholipid-dependent protein kinase activation by adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate in platelets. J Biochem (Tokyo) 91:403-406, 1982

28. Kaibuchi K, Takai Y, Ogawa Y, et al: Inhibitory action of adenosine 3′,5′-monophosphate on phosphatidylinositol turnover: Difference in tissue response. Biochem Biophys Res Commun 104:105-112, 1982

29. Vallar L, Spada A, Giannattasio G: Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas. Nature 330:566-568, 1987

30. Landis CA, Masters SB, Spada A, et al: GTPase inhibiting mutations activate the a chain of Gs and stimulate adenylate cyclase in human pituitary adenomas. Nature 340:692-696, 1989

31. Bourne HR, Stryer L: The target sets the tempo. Nature 358:541-543, 1992

32. Alvaro V, Levy L, Dubray C, et al: Invasive human pituitary tumors express a point mutated α-protein kinase-C. J Clin Endocrinol Metab 77:1125-1129, 1993