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Signal Transduction Pathways Employed by Thyrotropin-Releasing Hormone and Pituitary Adenylate Cyclase-Activating Polypeptide to Stimulate Prolactin Promoter Activity

by

Xiaohuai Chen

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1996

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Signal Transduction Pathways Employed by Thyrotropin-Releasing Hormone and Pituitary Adenylate Cyclase-Activating Polypeptide to Stimulate Prolactin Promoter Activity

by

Xiaohuai Chen

Adviser: Professor Carter Bancroft

Production of prolactin (PRL), a protein hormone secreted from the anterior pituitary, is regulated by a number of hypothalamic hormones. In my studies of hormonal action, I have concentrated on two of these: thyrotropin-releasing hormone (TRH) and pituitary adenylate cyclase-activating peptide (PACAP). As a model system, I have employed the GH3 line of rat pituitary cells. Both of these hormones have been shown previously to stimulate PRL promoter activity in these cells.

Exposure of GH3 cells to TRH has been shown to activate phospholipase C and increase the production of both diacylglycerol (DAG) and inositol triphosphate (IP3). DAG in turn leads to the activation of protein kinase C, and IP3 causes both the release of Ca^{2+} from intracellular stores and activation of Ca^{2+}/calmodulin kinase II activity. TRH has also been shown to stimulate phosphorylation of the oncogene *raf* and to activate mitogen activated protein kinase (MAP kinase) in these cells. Therefore, TRH stimulation of PRL promoter activity might be mediated by either one or a combination of these pathways. In my studies, I have employed pharmacological studies to provide evidence that TRH action is not mediated through either the PKC or the raf/MAP kinase pathways, but instead is mediated via a Ca^{2+}-Calmodulin-CaM kinase pathway. My studies also implicate the release of Ca^{2+} from intracellular stores in the transcriptional action of TRH.

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I then compared the signal transduction pathways employed by TRH and PACAP to regulate PRL promoter activity. Since one of the PACAP receptor subtypes expressed in GH3 cells can stimulate both adenylate cyclase and phospholipase C, PACAP stimulation of the PRL promoter could be mediated via a pathway involving either cAMP or inositol phosphates or both. First, I examined whether the effect of TRH and PACAP are additive. I found that optimal concentrations of TRH and PACAP have additive stimulatory effects on PRL promoter activity, implying that TRH and PACAP employ different pathways to regulate PRL promoter activity. My observation that both a dominant negative CREB mutant and mutation of a CRE-like element (CLE) in the PRL promoter partially block induction by either PACAP or forskolin (FSK), an adenylate cyclase activator, but do not block induction by TRH, further suggests that the signal transduction pathways employed by TRH and PACAP to regulate the PRL proximal promoter are different. These results also support the concept that PACAP acts on the PRL promoter via a cAMP/protein kinase A (PKA)-mediated pathway.

To investigate further the mechanism involved in PKA-mediated stimulation of PRL promoter activity, I examined whether a Pit-1 binding site in the PRL promoter (site 1P) can mediate PKA action. The observation that FSK stimulation can be directed by site 1P raises the possibility that Pit-1 may be involved in the PKA response. However, the observation that an unphosphorylatable Pit-1 mutant (Pit-1(A3)) can facilitate a response to FSK as strong as that with wild-type Pit-1 suggests that phosphorylation of Pit-1 is not required for PKA induction of PRL promoter activity. FSK was also observed to stimulate PRL promoter activity in 235-1(p-) cells, a Pit-1 deficient lactotroph cell line, suggesting that PKA can also induce PRL promoter in a Pit-1 independent manner. However, FSK and Pit-1 when added together, produce a marked synergistic stimulation of the PRL proximal promoter, indicating that the full effect of PKA requires involvement of Pit-1. This synergistic response was also not dependent on Pit-1

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phosphorylation, since substitution of Pit-1(A3) for Pit-1 did not reduce this synergism. Taken together, these results imply that PKA regulates PRL promoter activity via both Pit-1-dependent and Pit-1-independent mechanisms.

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Chapter I

Background

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I. Prolactin: An introduction

Prolactin, a 23 kDa single-chain protein hormone which can stimulate breast development and milk production and influence reproductive function, is released by mammotropic cells from anterior pituitary. In women, prolactin deficiency results in the inability to lactate, whereas overproduction may cause infertility. Because of prolactin's important role in physical reproduction, it is important to elucidate the mechanism(s) responsible for the regulation of both prolactin gene expression and prolactin secretion.

The hypothalamus regulates the hormone producing activity of the anterior pituitary by releasing neurohormones that are delivered to the anterior pituitary via the hypothalamohypophyseal portal system. The hypothalamohypophyseal portal system provides a means by which the hypothalamus, using neurohormones as chemical signals, can regulate the secretory activity of the adenohypophysis. The pituitary gland, roughly one cm in diameter, is divided functionally into two parts: the neurohypophysis (posterior pituitary) and the adenohypophysis (anterior pituitary). The anterior pituitary contains at least five endocrine cell types: corticotropes, thyrotropes, gonadotropes, somatotropes, and mammotropes. These different cell types produce a number of protein hormones which regulate numerous physical functions, including the secretory activity of several other endocrine glands. These hormones include: thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH) and prolactin (PRL). PRL levels are regulated by hypothalamic factors. For example, thyrotropin-releasing hormone (TRH) stimulates PRL secretion and synthesis, whereas dopamine inhibits both its synthesis and release. A number of other hormones and factors, including Ca^{2+}, pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), epidermal growth factor (EGF) and estrogen, can also influence PRL secretion and synthesis. In recent years,

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research on the regulation of PRL expression has progressed very rapidly. As described further below, the transcription factor Pit-1 has been found to play an important role in the regulation of PRL gene expression.

II. Role of the transcription factor Pit-1 in cell type specific expression of the prolactin and growth hormone genes

In 1988, a 31-33 kDa, pituitary specific transcription factor Pit-1 (also termed GHF-1) was cloned (Ingraham et al., 1988; Bodner et al., 1988) and shown to activate both the PRL and GH genes (Ingraham et al., 1988; Fox et al., 1990; Mangalam et al., 1989; Simmons et al., 1990). Overexpression of Pit-1 in GH3 cells, a cell line derived from rat pituitary tumor cells that makes and secretes PRL and GH, can increase the transcription of both PRL and GH (Mangalam et al., 1989). Expression of Pit-1 in non-pituitary cell lines, which do not express Pit-1, also induces the expression of the PRL and GH genes (Ingraham et al., 1988; Fox et al., 1990; Mangalam et al., 1989). Pit-1 belongs to the POU-homeodomain family of homeobox proteins, where the term POU comes from the first letter of four transcription factors, Pit-1, Oct-1/Oct-2 and Unc-86, each of which contains this POU homology amino acid sequence (Herr et al., 1988).

Pit-1 is routinely seen as a 31-32 kDa doublet in protein gels, resulting from the alternative use of translation initiation sites. It has been shown that both the 31 kDa- and 33 kDa Pit-1 proteins transactivate the prolactin gene equally (Voss et al., 1991).

III. Structure of transcription factor Pit-1

Pit-1 contains three functional regions (see **Fig. 1.1**): (**1**) the transactivation domain (amino acid residues 8-80), which is involved in activating the target genes (Theill et al., 1989; Ingraham et al., 1990; Ding et al., 1991), (**2**) the POU-specific domain (amino acid residues 128-198), which confers high-affinity sequence-specific

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DNA binding and protein interaction (Verrijzer et al., 1992; Ingraham et al., 1990), (3) the POU homeodomain (amino acid residues 213-273), which is necessary but not sufficient for high affinity DNA binding (Ding et al., 1991). Structural analysis of Pit-1 has revealed two α helices in the POU-specific domain, and three in the POU-homeodomain. In the POU homeodomain, the second and third helices form a structure similar to the helix-turn-helix motif found in many prokaryotic repressors. Pit-1 has been shown to be phosphorylated in rat pituitary cells (GC cells) in response to either cAMP or phorbol ester treatment. Phosphorylation alters the binding of Pit-1 to its response elements in both the PRL promoter and the Pit-1 promoter (Kapiloff et al., 1991). *In vitro* experiments have shown that protein kinase A (PKA) induces Pit-1 phosphorylation at three sites (see **Fig. 1.1**): Ser^{115}, Thr^{220} and Thr^{219} (Kapiloff et al., 1991).

IV. Structure of the rat PRL promoter

The activity of the PRL gene is influenced by two regions, a proximal promoter region and a distal enhancer region. The proximal promoter region contains the first approximately 200 bp 5' to the transcription start site, whereas the enhancer region is located ~1500 bp upstream of the transcription start site (Lufkin and Bancroft, 1987; Lufkin et al., 1989; Day and Maurer, 1989; Nelson et al., 1988). Pit-1 has been shown to bind four sites (1P, 2P, 3P and 4P) in the rat PRL proximal 5'-flanking DNA region (Lufkin and Bancroft, 1987; Lufkin et al., 1989; Gutierrez-Hartmann et al., 1987) and to another four sites (1D, 2D, 3D, and 4D) in the distal promoter region (See **Fig. 1.2**). Either region is sufficient to direct the expression of reporter genes in pituitary cells of transgenic animals, but both regions are required for high level expression that is confined to lactotropes (Crenshaw III et al., 1989).

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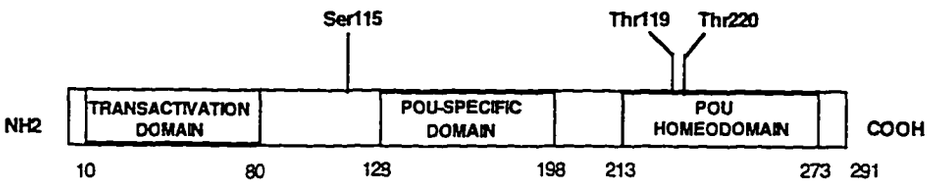

Functional domains of Pit-1 protein and its possible phosphorylation sites.

Figure 1.1

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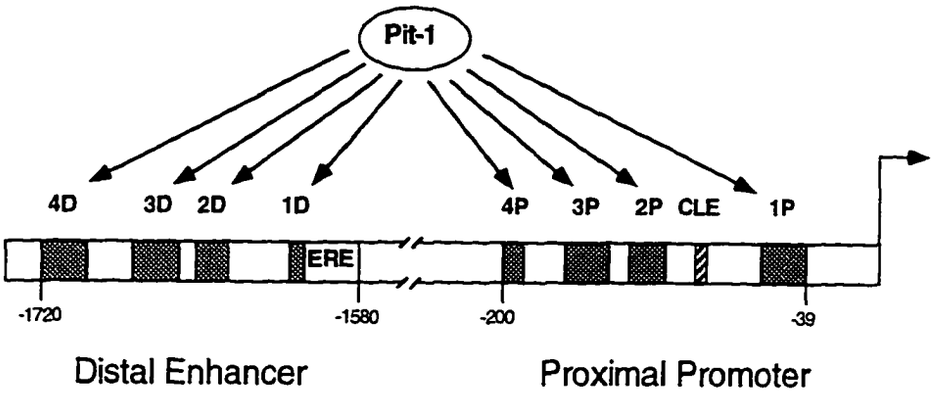

Pit-1 binding sites in the prolactin 5' upstream flanking sequence.

Figure 1.2

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Besides Pit-1 binding sites, the distal rat PRL promoter region also contains an estrogen receptor binding site, located immediately adjacent to Pit-1 binding site 1D (Waterman et al., 1988). The sequence of the estrogen receptor (ER) binding site resembles the palindromic consensus ER-binding site, but is not a palindrome (Maurer and Notides, 1987). A mutation that disrupts this imperfect palindrome abolishes estrogen induction of the PRL gene (Kim et al., 1988).

The proximal rat PRL promoter region also contains a cAMP-response element-like element (CLE), located between 1P and 2P of the PRL promoter. CLE contains a TGACG motif similar to the consensus ATF/CREB-binding site found in many cAMP-regulated promoters. A mutation in CLE strongly reduces not only the level of cAMP regulation but also the Ca^{2+} regulation and basal activity of the hPRL promoter (Peers et al., 1992). CLE was shown to bind to a ubiquitous factor, which is not CREB and has a molecular mass of approximately 100 kDa (Peers et al., 1992). Unfortunately, this protein has yet to be cloned.

V. Introduction to thyrotropin releasing hormone and pituitary adenylate cyclase-activating peptide

Thyrotropin releasing hormone (TRH) is a tripeptide (pyro-Glu-His-Pro-NH2) that was originally isolated from hypothalamic extracts (Burgus et al., 1970; Nair, 1970). TRH stimulates the production of thyroid stimulating hormone (TSH) and prolactin in the anterior pituitary (Vale, 1977) by regulating both their secretion and transcription.

Pituitary adenylate cyclase-activating peptide (PACAP) is a recently purified member of the vasoactive intestinal peptide (VIP)-secretin-glucagon family (Miyata, 1989) (Miyata et al., 1990). The PACAP precursor is processed into two peptides (Kimura, 1990; Ogi et al., 1990; Ohkubo et al., 1992): PACAP-38, which is the major form of PACAP, and PACAP-27, which corresponds to the N-terminal 27 residues of PACAP-

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38 (Arimura et al., 1991). The N-terminal sequence of PACAP-38 shares a 68% amino acid homology with VIP (Miyata, 1989). Although the precise physiological role of PACAP is yet to be determined, the following evidence suggests that PACAP is a modulator of pituitary functions: (1) PACAP is found in both the hypothalamus and the pituitary, including the median eminence which is in close contact with the hypophyseal portal capillaries (Arimura, 1992); (2) PACAP receptors are present on anterior pituitary cell membranes (Gottschall et al., 1990), and both PACAP type I and type II receptors are expressed in GH3 cells (Coleman et al, 1996); (3) PACAP has been shown to induce and/or potentiate the release of growth hormone, prolactin, adrenocorticotropic hormone (ACTH), luteinizing hormone, follicle-stimulating hormone and α-melanocyte-stimulating hormone (Miyata, 1989; Culler and Paschall, 1991; Goth et al., 1992; Hart et al., 1992; Koch and Lutz, 1992; Propato et al., 1992); (4) PACAP stimulates the production by pituitary cells of both cAMP (Coleman and Bancroft, 1993) and cellular inositol phosphate (Spengler et al., 1993); (5) PACAP stimulates PRL promoter activity in GH3 cells (Coleman and Bancroft, 1993) and increases expression of growth hormone mRNA in cultured pituitary cells (Velkeniers et al., 1994).

The cellular actions of PACAP and TRH are described in more detail in the introductions to Chapters II and III, respectively.

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Chapter II

TRH Action on the Prolactin (PRL) Promoter Requires Ca^{2+}/Calmodulin-Dependent Protein Kinase (CaM Kinase) Activity

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INTRODUCTION

Early studies showed that TRH stimulates both the release and synthesis of prolactin from pituitary cells and increases prolactin mRNA levels in these cells (Vale, 1977; Martin et al., 1983; Murdoch et al., 1982). Further studies showed that the PRL promoter contains at least two TRH response elements (TRHREs) (Yan et al., 1991): One is located between position -75/+38 which was identified as site 1P, the other may be element 3P (Yan et al., 1991). Pit-1, which binds these two sites, has been shown to be a mediator of TRH action on the PRL promoter (Yan et al., 1991). In addition, Ca^{2+} mediates TRH action on the PRL proximal promoter (Yan et al., 1991). Despite these findings, much remains to be investigated concerning the precise mechanism(s) by which TRH regulates transcription of the PRL gene. In this section, I will discuss the pathways which are believed to be involved in cellular actions of TRH and regulation of the PRL promoter.

1. Extranuclear actions of TRH

The TRH receptor can activate phospholipase C and protein kinase C

In 1990, the TRH receptor (TRHR) was cloned from mouse pituitary (Straub et al., 1990). Analysis of the cloned cDNA sequence indicated that the TRHR belongs to the putative seven transmembrane domain, G protein coupled receptor family. Further evidence showed that TRHR is coupled to the G proteins $G\alpha_q$ and/or $G\alpha_{11}$ (Aragay et al., 1992) and can activate phospholipase C (PLC) (Hsieh and Martin, 1992). PLC then causes an increase in the production of 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Martin, 1983; Rebecchi and Gershengorn, 1983; Rebecchi et al., 1983). The DAG produced further leads to the activation of protein kinase C (PKC)

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(Drust and Martin, 1984). Following TRH addition, IP3 production reaches maximal levels within five seconds (Martin, 1983; Drummond et al., 1984). The IP3 produced can cause release of Ca^{2+} from the endoplasmic reticulum by binding to IP3 receptors on the endoplasmic reticulum membrane (see **Fig. 2.1**) (Tsien, 1990).

The TRH receptor can increase cytosolic free Ca^{2+} via both voltage-dependent Ca^{2+} channels and IP3 receptors

It was reported that TRH stimulates a biphasic elevation of cytoplasmic free Ca^{2+} in GH3 cells (Albert and Tashjian, 1984). The first phase of this elevation is largely resistant to the presence of the Ca^{2+} channel blockers nifedipine and verapamil, which markedly reduce the second phase (Albert and Tashjian, 1984; Gershengorn and Thaw, 1985). Furthermore, the removal of extracellular Ca^{2+} shortly before the addition of TRH has little effect on the initial spike of Ca^{2+}, but seems to prevent the subsequent "plateau" phase (Albert and Tashjian, 1984; Gershengorn and Thaw, 1983). These data suggest that most , if not all, of the first phase of the Ca^{2+} signal derives from intracellular sources, while the second phase is due largely to influx of extracellular Ca^{2+} through Ca^{2+} channels.

It has been reported that in GH3 cells, TRH transiently inhibits voltage-dependent Ca^{2+} channels, presumably because of a direct action of Ca^{2+} (Gollasch et al., 1991; Levitan and Kramer, 1990). However, under Ca^{2+} free conditions, which also empties the IP3-sensitive intracellular Ca^{2+} stores, TRH stimulates Ca^{2+} channel activity (measured as sodium current through voltage-dependent Ca^{2+} channels). By intranuclear injection of antisense oligonucleotides, a pertussis toxin sensitive G protein Gi2 was found to be involved in TRH action on Ca^{2+} channels (Gollasch et al., 1993). In addition, requirement for a concurrent hormone-induced phosphoinositol response and consequent PKC activation was demonstrated by either antisense

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oligonucleotide-induced suppression of the Gq/G11 α subunit or treatment of GH3 cells with PKC inhibitors (Gollasch et al., 1993).

TRH can activate Ca^{2+}/calmodulin-dependent protein kinase II activity in GH3 cells

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) was originally discovered in rat brain as a Ca^{2+}-dependent protein kinase (Stulman and Greengard, 1978; Yamauchi and Fujisawa, 1980). Its substrates include (**1**) enzymes, such as tyrosine hydroxylase, (**2**) cytoskeletal proteins, such as synapsin 1, MAP-2, tau and tubulin, and (**3**) transcription factors, such as CREB and C/EBPβ (Kemp, 1988; Sheng et al., 1991; Dash et al., 1991; Wegner et al., 1992).

CaM kinase II initially requires Ca^{2+}/CaM for its activity, but as Ca^{2+} levels increase, it undergoes phosphorylation and also phosphorylates its substrates (Lou and Schulman, 1989). Autophosphorylation greatly enhances the binding between CaM kinase II and calmodulin, allowing CaM kinase II to maintain 85% of its initial activity even when cellular Ca^{2+} is at basal levels (autonomy) (Meyer, 1992).

Early in 1983, Hatada et al. (Hatada et al., 1983) identified CaM kinase activity in rat anterior pituitary gland. In 1991, Jefferson et al (Jefferson et al., 1991) found that the rat pituitary cell line GH3 contains a CaM kinase very similar to the rat brain CaM kinase. Immunoprecipitation of GH3 cell extracts using a monoclonal antibody selective for the neuronal CaM kinase showed that the CaM kinase in GH3 cells consists of at least two peptides of 52-54 kDa (Jefferson et al., 1991). Stimulation of GH3 cells with physiological levels of TRH elicited a rapid increase in CaM kinase activity, followed by a low level of autonomy which lasts for several minutes. Upon TRH and K^+ stimulation of the GH3 cells, CaM kinase is phosphorylated at sites which correspond to

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the characterized autonomy sites of rat brain CaM kinase and becomes Ca^{2+}-independent (Jefferson et al., 1991).

TRH can activate MAP kinase activity in GH3 cells

It has recently become apparent that signals arising from the growth factor receptor tyrosine kinase family result in the activation of a family of intracellular serine/threonine kinases referred to as the mitogen activated protein kinases (MAP kinases). In this pathway, the receptors are indirectly coupled to the p21 ras protooncogene, followed by the downstream stimulation of raf-1 kinase, MAP kinase kinase and MAP kinase (Blenis, 1993; Pelech and Sanghera, 1992). In 1993, Ohmichi et al. reported that TRH can induce the accumulation of the GTP-bound form of p21 ras, increase the phosphorylation of raf-1, and activate MAP kinase in GH3 cells. Maximal activation of MAP kinase was detected after a 5 min exposure to TRH. Down regulation of cellular protein kinase C (PKC) only partially inhibited the phosphorylation of MAP kinase induced by TRH, suggesting the involvement of both PKC-dependent and PKC-independent pathways (Ohmichi et al., 1994).

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2. Pathway of action of TRH on the PRL promoter

TRH stimulation of prolactin gene expression may not require protein kinase C activity

Previous work showed that TRH can activate PKC and elevate intracellular Ca^{2+}, which functions as a required cofactor of PKC (Nishizuka, 1984). Further evidence showed that TRH treatment induces phosphorylation of a series of cytoplasmic proteins in a Ca^{2+}- and PKC-dependent manner (Drust and Martin, 1984). The phorbol 12-myristate 13-acetate (TPA), an activator of PKC, directly stimulates expression of the PRL gene (Kaji et al., 1988). Therefore, it seemed possible that PKC may mediate TRH-induced PRL gene expression (see **Fig. 2.1**).

But preliminary results in Bancroft's lab showed that down regulation of protein kinase C by chronic TPA exposure did not block either Ca^{2+} stimulation of PRL mRNA levels (Bandyopadhyay and Bancroft, 1989), or TRH stimulation of PRL promoter activity (Yan and Bancroft, 1991). These results implied that PKC activity is not required for TRH- or Ca^{2+} action on PRL gene expression. My results described in this chapter further support this conclusion.

Ca^{2+}/calmodulin kinase is a candidate for mediating the TRH transcriptional signal transduction pathway

It has been reported that upon TRH stimulation, cytosol Ca^{2+} levels are rapidly raised by IP3 stimulated Ca^{2+}-release from intracellular pools, followed by a sustained influx of extracellular Ca^{2+} via a voltage-dependent Ca^{2+} channels (Gershengorn and Thaw, 1985; Hagiwara and Ohmori, 1983; Tan and Tashjian, 1984). The increased intracellular Ca^{2+} then binds to calmodulin, causing the activation of Ca^{2+}/calmodulin

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dependent protein kinases, which phosphorylate their downstream substrates (Schulman, 1993). Recent evidence shows that upon TRH stimulation, CaM kinase II activity is increased in GH3 cells (Jefferson et al., 1991).

TRH-stimulated PRL expression is dependent on Ca^{2+}

White et al. (1981) (White et al., 1981) reported that in addition to its role in PRL secretion, Ca^{2+} can specifically stimulates PRL synthesis at a nuclear pretranslational level in GH3 cells. Further investigation indicated that in the presence of the Ca^{2+} chelator EGTA, TRH produces no detectable stimulation of PRL mRNA levels, while TRH strongly stimulates PRL mRNA levels in the presence of 0.4 mM Ca^{2+} (White and Bancroft, 1983). There is also evidence that cobalt ions, which have been shown to inhibit Ca^{2+}-mediated effects both at level of influx of extracellular Ca^{2+} and at intracellular locus (Thaw et al., 1984), block the induction of the PRL proximal promoter by either TRH or Ca^{2+} (Yan and Bancroft, 1991).

Yan and Bancroft also reported that nimodipine, a voltage-dependent Ca^{2+} channel inhibitor which blocks Ca^{2+} fluxes during depolarization (Lee and Tsien, 1983; Janis, 1987), which occurs spontaneously in GH3 cells and are caused by Ca^{2+} action potentials (Taraskevitch, 1980), can also inhibit TRH-induced PRL gene expression (Yan and Bancroft, 1991). This result implies that a voltage-dependent Ca^{2+} channel may also be involved in the action of TRH on the PRL promoter.

Yan and Bancroft found that the first 75 bp of the PRL promoter region which contains the Pit-1 binding site 1P can confer Ca^{2+} induction (Yan and Bancroft, 1991). Since the Pit-1 binding site 1P has been shown to be one of the TRH response elements (Yan et al., 1991), these data implied that Ca^{2+} is involved in mediating TRH action on PRL gene expression.

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Calmodulin (CaM) may be involved in TRH stimulation of PRL gene expression

Calmodulin is a ubiquitous, multifunctional Ca^{2+} binding protein with a molecular weight of 17 kDa. It is a symmetrical dumbbell-shaped molecule with two Ca^{2+}-binding domains on each end connected by an α-helical structure (Babu, 1985). It possesses four Ca^{2+} binding sites, two of which are high affinity binding sites, while the other two are low affinity sites (Cox, 1988).

Bancroft and White (1984) reported that N-(6-aminohexyl)-5-chloro-naphthalenesulfonamide·HCL (W7), a calmodulin inhibitor, can inhibit PRL expression stimulated by either TRH or EGF in the presence of Ca^{2+} (White and Bancroft, 1984). Davis et al (1991) reported that W7 reduces PRL mRNA levels and prevents stimulation of the PRL promoter by TRH (Davis et al., 1991). These results suggest that calmodulin, the ubiquitous Ca^{2+} binding protein, may be involved in both basal and TRH induced PRL expression. Since W7 is relatively non-specific in its action, I have employed more specific calmodulin inhibitors to investigate this question.

Many calmodulin-regulated processes are mediated by the Ca^{2+}/calmodulin dependent protein kinases (CaM kinases). Since CaM kinase II has been shown to be activated by TRH in GH3 cells (Jefferson et al., 1991), it is possible that Ca^{2+}/calmodulin dependent kinase II may be involved in TRH transcriptional pathway.

As discussed above, TRH can activate the PLC/PKC pathway, a Ras/Raf pathway, as well as CaM kinase pathways (see **Fig. 2.1**). To determine the exact pathway mediated by TRH, I examine the abilities of different inhibitors for these pathways to block TRH stimulation of the PRL promoter. In this chapter, I present evidence that TRH action on the PRL promoter requires CaM kinase activity.

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Possible cellular pathways for TRH action on the prolactin promoter

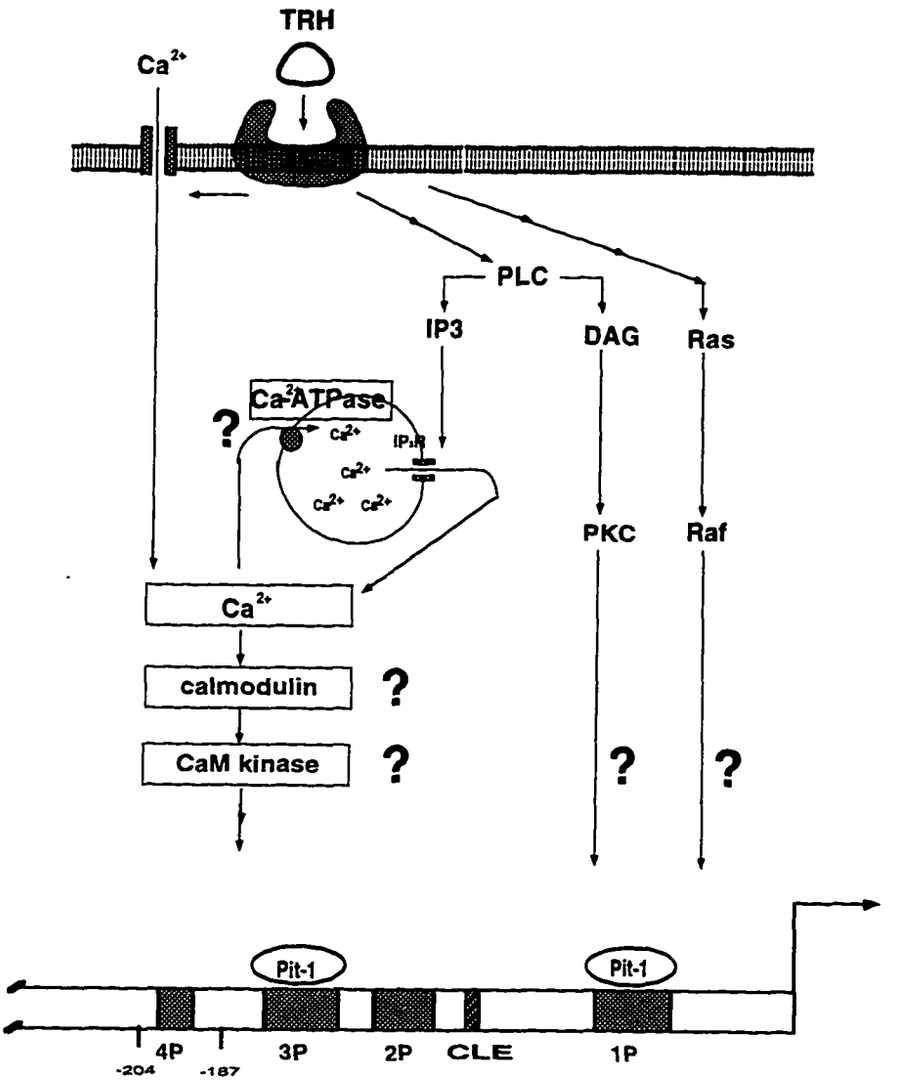

Figure 2.1

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MATERIALS AND METHODS

Materials

GH3 cells were obtained from the American Type Culture Collection (Rockville, MD). TRH, forskolin (FSK) and phorbol ester (PMA) were obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS), horse serum, penicillin, streptomycin, and trypsin were from Gibco/BRL (Gaithersburg, MD). [^{3}H]-chloramphenicol was from Du Pont NEN (Boston, MA). Butyl Coenzyme A was from the Sigma Chemical Company (St. Louis, MO) or Fisher Scientific Co (Pittsburgh, PA). Calphostin C was provided by Dr. Jay Unkeless (Biochemistry Department, Mt. Sinai). Rp-cAMP was supplied by BioLog (La Jolla, CA). All other inhibitors were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

Constructs

The PRL promoter-CAT reporter construct (-187)PRL-CAT has been described previously (Lufkin et al., 1989). pTK-GH was part of the kit used for assays of HGH for corrections for transfection efficiency (Nichols Institute, San Juan Capistrano, CA). Rc/RSV was obtained from Invitrogen Corporation (San Diego, CA).

pRSV-RafC4B, a plasmid expressing a dominant negative Raf gene, which bears a deletion of the carboxy-terminal catalytic domain, and pRSV-Raf-C4pm17B, a mutant of Raf-C4B which is no longer dominant negative, were kindly provided by Dr. U. Rapp. These two constructs are the same as the construct pRSV-C4 and pRSV-C4pm17 (Bruder et al., 1992), except for the inclusion of a C-terminal B-Raf antigen tag, which does not influence their regulatory properties (U. Rapp, personal communication).

An expression vector for dominant negative Ras, pSV2Ras, was provided by Dr. Thomas Y. Shih (Ogiso et al., 1993). The dominant negative Ets construct, pAPrEts-Z,

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which lacks the amino-terminal transactivation domain, encoding only the consensus DNA-binding domain and nuclear localization signal of Ets-2, was kindly provided by Dr. M. Ostrowski (Lange et al., 1992).

Cell culture and transfection in suspension

GH3 cells were grown in suspension cultures in Minimum Essential Medium (Joklik modified) with 10 mM HEPES, pH 7.4, 25 mM NaHCO3, 100 mg/ml streptomycin, 100 U/ml penicillin, 2.5% fetal calf serum, and 15% horse serum. I modified the suspension transfection method for T lymphocytes (Fujita, 1986) for use with GH3 cells. Briefly, for each transfection, 1.2 X 10^7 GH3 cells were spun down and washed in 10 ml STBS (25 mM Tris-Cl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na2HPO4, 0.7 mM CaCl2, 0.5 mM MgCl2). The cells were resuspended in 1 ml of STBS containing 0.4 mg DEAE-dextran. Following the addition of the indicated amounts of plasmid DNA constructs, the cells were incubated at 37°C for 40 min. DMSO (100 ml) was then added dropwise. Following incubation for 3 min at 37°C, the cells were washed once with STBS, once with DMEM, resuspended in DMEM containing 5% fetal calf serum, and plated at a density of 2 X 10E6/per well on 6-well Falcon plate. When multiple transfections with the same plasmid(s) were carried out, the transfected cells were pooled prior to plating onto separate dishes, in order to normalize the transfection efficiency in all dishes. Following plating, the cells were incubated for 20 hours at 37°C, 5% CO2.

Treatment with hormones and preparation of cell extracts

Twenty hours after transfection, the medium was aspirated, and replaced with serum free medium (SFM) containing 0.5 mM Ca^{2+} (Bauer, et al., 1976; White et al., 1987). Following deinduction in SFM for two days, cell were treated with various inhibitors for three hours, followed by addition of stimulators as necessary. Eighteen hours after the addition of stimulators, cells were harvested, and sonicated as previously described

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(Yan et al., 1991). Briefly, cells from each well were collected into individual microfuge tubes using a cell scraper. The cells were then pelleted for 10 seconds at 16,000xg, resuspended in 100 µl of CAT buffer (250 mM Tris-HCL pH 7.8, 10 mM EDTA), and sonicated on ice for eight minutes (2X4 min) in a Fish Sonic Dismembrator at 90% power. The cellular extracts were then incubated at 65°C for five minutes to inactivate cellular deacetylases, followed by centrifugation for five minutes at 16,000xg. The supernatants were saved and stored at -20°C.

Measurement of CAT activity and correction for transfection efficiency

CAT activity was assayed by the phase extraction technique (Fischberg et al., 1994; Seed and Sheen, 1988). Briefly, 50 µl of cellular extract (one-half of the extract from each well) was mixed with 10 µl 1 M Tris-HCl pH 8.0, 20 µl 0.01 µCi/µl [^{3}H] chloramphenicol, 5 µl butyl Coenzyme A (5 mg/ml) and 15 µl H_2O, and incubated for 4 to 18 hours at 37°C. Following incubation, the reaction mixture were extracted with 200 µl of TMPD/Xylene solution (2:1 tetramethylpentadecane/xylene). The top organic phase (150 µl) containing the acylated product was collected and mixed with Ecolite(+) scintillation fluid. Scintillation counting was then performed on a Beckman LS-1801 counter. Reaction mixtures incubated with extracts of untransfected cells were used to obtain background values which were subtracted from all experimental values.

When different plasmids were used in the same transfection experiment, possible variations in transfection efficiency were corrected for by cotransfection of plasmid pTK-GH. Human growth hormone in the medium was then assayed with the HGH-TGES transient gene expression kit (Nichols Institute, San Juan Capistrano, CA), and the results used to normalize the results of the CAT assays.

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Data analysis and statistics

All experiments were performed in either duplicate or triplicate as indicated in each figure legend. Each figure is representative of the results of several independent experiments, as indicated in the figure legends. Data are presented as mean+/-SD.

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RESULTS

As I described in Chapter I, activity of the PRL gene is influenced by two DNA regions, a proximal promoter region and a distal enhancer region. Either region is sufficient to direct the expression of reporter genes in pituitary cells of transgenic animals, but both regions are required for high level expression that is confined to lactotrophes (Crenshaw III et al., 1989). The proximal promoter region contains the first approximately 200 bp 5' to the transcription start site. (Lufkin and Bancroft, 1987; Lufkin et al., 1989; Day and Maurer, 1989; Nelson et al., 1988). Since it has been previously shown that the first 187 base pairs of the PRL promoter can direct a complete TRH response (Lufkin et al., 1989), I used (-187)PRL-CAT as a reporter construct in all of the experiments discussed in this chapter.

1. TRH stimulates PRL promoter activity via a pertussis toxin-insensitive mechanism

G proteins are very important signal transducers that control biological functions. Twenty different G protein subunits have been found, which can be divided into four subfamilies (Devivo and Iyengar, 1994). G proteins play essential roles in many intracellular signaling pathways. Their roles include the activation or inhibition of adenylate cyclase, activation of ion channels and activation of PLC. It has been shown that the TRHR is coupled to both the pertussis toxin (PTX)-sensitive Gi2 subunits (Gollasch et al., 1993) and the PTX-insensitive $G\alpha_q$ and/or $G\alpha_{11}$ subunits (Gershengorn, 1993). To begin the investigation of the possible involvement of G proteins in TRH action on the PRL promoter, I examined the ability of PTX to block this action. As shown in **Fig. 2.2**, PTX does not block TRH stimulation of the PRL

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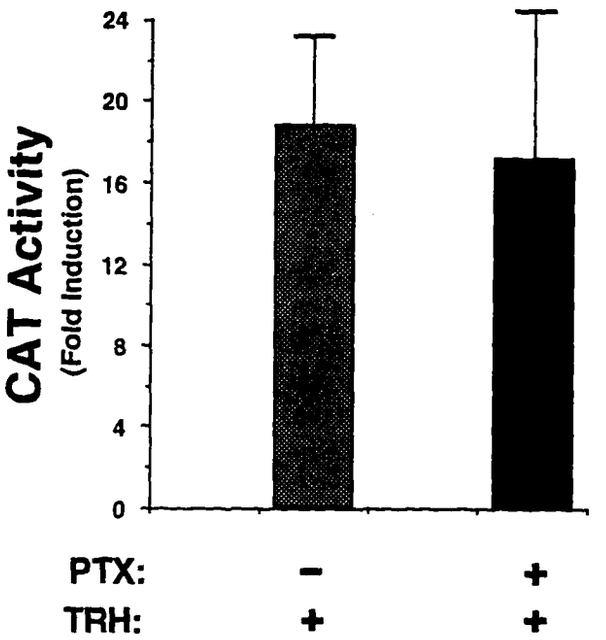

Pertussis toxin (PTX) does not block TRH stimulation of PRL promoter activity. GH3 cells ($1.2X10^7$) were transfected in suspension with 10 μg (-187)PRL-CAT, divided, and plated into 35 mm dishes ($2X10^6$ cells/dish). Following deinduction (see "Materials and Methods"), the cells were incubated three hours ± pertussis toxin (PTX) (200 ng/ml), 18 hours ± TRH (100 nM), and assayed for CAT activity. Shown is TRH stimulation (fold ± SD) observed with triplicate dishes.

Figure 2.2

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promoter, although PTX does decrease basal expression of PRL promoter (data not shown). This observation suggests that PTX-sensitive G protein subtypes are not involved in TRH induction of PRL promoter activity.

2. TRH stimulation of PRL promoter activity is not blocked by inhibitors of either protein kinase C or protein kinase A

Since TRH can activate PKC via phospholipase C, it seems possible that TRH action might be mediated by PKC. However, Yan et al (Yan and Bancroft, 1991) found that down-regulation of PKC in GH3 cells by chronic exposure to TPA had no effect on TRH-induced PRL-CAT activity. To further investigate this unexpected result, I examined whether either of two PKC inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) (Hidaka, 1984) or Calphostin C (Kobayashi, 1989), could block TRH activation of the PRL promoter.

It is known that pharmacological inhibitors can exhibit non-specific effects on cellular kinases. For example, H-7 is almost as effective against protein kinase A (PKA) as against PKC (Hidaka et al., 1984). Thus, in all my studies of this type, I have used internal controls, both to show that the inhibitors are active under the conditions employed, and to investigate inhibitor specificity.

Previous work has shown that both TPA (Jackson et al., 1990), a PKC activator, and forskolin (Keech et al., 1992), an adenylate cyclase stimulator, can stimulate PRL gene expression. To evaluate the specificity of H-7 on TRH stimulated PRL gene expression, I also examined the ability of H-7 to block TPA and FSK induction of PRL-CAT activity. **Figure 2.3** shows that H-7 moderately inhibited induction of the PRL promoter by FSK, while completely inhibiting induction by TPA. These results show that H-7 blocks PKC activity, but only partially inhibits PKA activity under the experimental conditions I have employed. The observation that H-7 does not inhibit

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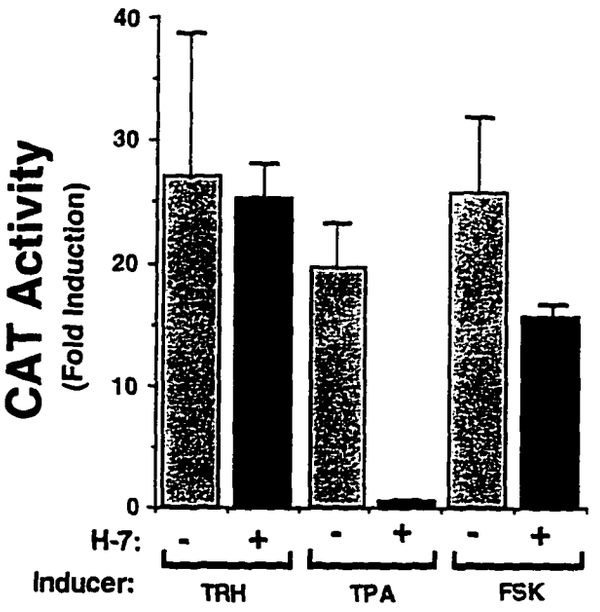

The protein kinase C (PKC) inhibitor H-7 does not inhibit TRH stimulation of PRL promoter activity. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated three hours± H-7 (20 μM), 18 hours ± TRH (100 nM) or TPA (100 nM) or FSK (1 μM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by TRH, TPA, or FSK observed with triplicate dishes.

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TRH induction (**Fig. 2.3**) implies that the TRH transcriptional pathway on the PRL promoter is not mediated by protein kinase C.

To confirm these observations, I conducted similar studies using another highly specific PKC inhibitor, Calphostin C (**Fig. 2.4**). It is seen that Calphostin C specifically inhibits induction of PRL promoter activity by TPA, while having no effect on either TRH or FSK stimulation of PRL-CAT activity. These results further support the conclusion that PKC is not involved in TRH action on PRL transcription.

These data also suggest that PKA activity is not required for TRH-induced PRL gene expression. This would agree with a previous report showing that TRH receptor activation does not elevate cAMP levels in cells expressing high levels of TRH receptors, including GHY, AtT-20, or Hela cells (Heinflink et al., 1994). To further test this concept, I performed similar preliminary studies using both N-[2-(2-Bromocinnamylomino)ethyl]-5-isoquinoline-sulfonamide·2HCL (H-89), a potent inhibitor specific for PKA (Nishizuka, 1986), and Rp-cAMP, a nonhydrolizable form of cAMP and the most specific PKA inhibitor known (Maldonado and Schumann, 1991). I found that neither H-89 nor Rp-cAMP can block TRH action on the PRL promoter (data not shown). Consistent with these observations, KCREB, a dominant negative form of CREB, was found to be unable to block TRH induction of the PRL promoter (see **Chapter III** below).

3. Evidence that TRH induction of PRL promoter activity is not mediated by a MAP kinase pathway

Gutierrez-Hartmann has shown that constitutively active Ras or Raf can activate PRL promoter activity (Conrad and Gutierrez-Hartmann, 1992), and that the nuclear acceptor of the Ras response includes a member of the Ets family of transcription factors (Conrad et al., 1994). Further investigation showed that the proximal PRL promoter contains

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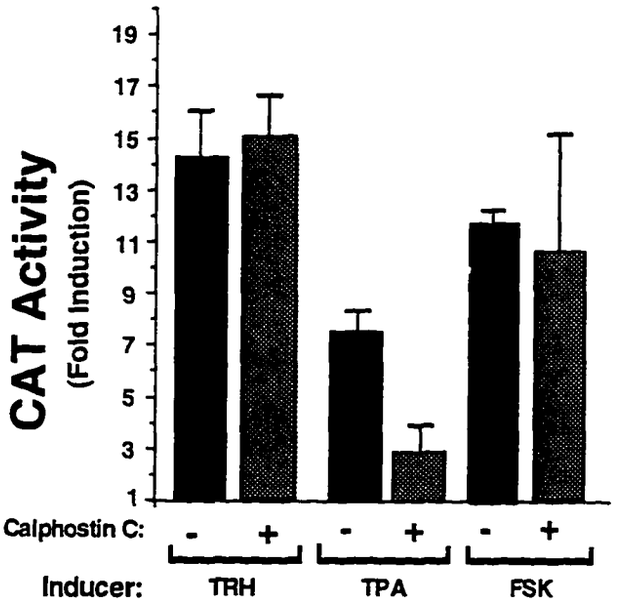

The PKC inhibitor calphostin C does not inhibit TRH stimulation of PRL promoter activity. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated three hours ± calphostin C (1.2 μM), 18 hours ± TRH (100 nM) or TPA (100 nM) or FSK (1 μM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by TRH, TPA, or FSK observed with triplicate dishes.

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several putative Ets consensus-binding sites, 5' (A/C)GGAA-3' (Macleod, 1992; Wasylyk et al., 1993). These findings raise the possibility that TRH action may be mediated by the Ras-Raf kinase-MAP kinase-Ets pathway. To investigate this possibility, I examined whether TRH induction can be blocked by a dominant negative mutant of Raf. The Raf-C4B mutant (Bruder et al., 1992) encodes only the amino terminal cysteine finger regulatory domain of the c-raf-1 protein, which is responsible for binding to Ras. Raf-C4pm17B is a mutant of Raf-4b which contains a cysteine to serine substitution in the cysteine finger motif of the amino-terminal regulatory domain, making Raf-C4pm17B no longer inhibitory (Bruder et al., 1992). Expression vectors for dominant negative Raf (Raf-C4B) or for the mutated dominant negative Raf (Raf-C4pm17B) (Bruder et al., 1992) were cotransfected with (-187)PRL-CAT into GH3 cells and then incubated with TRH. I found that either Raf-C4B nor Raf-C4pm17B blocks TRH induction (**Fig. 2.5**), although both decrease basal expression (data not shown). These results raise the possibility that TRH action is not mediated by Raf.

In preliminary experiments, I also examined the ability of plasmids expressing either dominant negative Ras, pSV2ras116r, or a dominant negative Ets, pApr-ets, to block TRH induction. I found that neither construct blocked TRH induction, but they both slightly decrease basal expression (data not shown). Based on these observatons, it seems unlikely that the transcriptional effect of TRH is mediated by the Ras-Raf-MAP kinase pathway. To make a solid conclusion concerning this issue, further investigation is still necessary (see Discussion).

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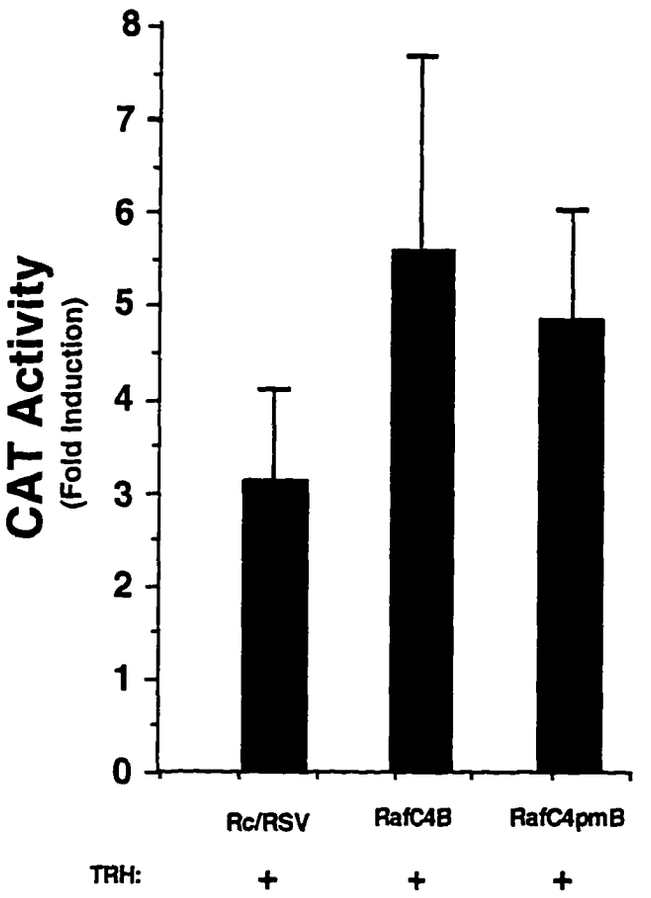

A dominant negative Raf mutant, RafC4B, does not inhibit TRH stimulated PRL gene expression. GH3 cells were transfected with 10 μg (-187)PRL-CAT and 2.5 μg tk-GH plus 5 μg Rc/RSV or RSV-rafC4B (rafC4B) or RcRSV-rafC4 pm17B (rafC4pmB), divided, and plated as described in Fig. 2.2.. Following deinduction, the cells were incubated 18 hours ± TRH (100 nM), and assayed for CAT activity. Shown is TRH stimulation (fold ± SD) observed with triplicate dishes. Transfection efficiencies were normalized as described in Material and Methods.

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4. Inhibitors of either Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II) or calmodulin inhibit TRH stimulation of PRL promoter activity

As discussed earlier, TRH action on PRL gene expression is mediated by Ca^{2+}. However, as described above, the observed effects of Ca^{2+} are apparently not mediated by PKC. We, therefore, decided to investigate whether other Ca^{2+} activated protein kinases might be responsible for these effects. Recent evidence which showed that TRH can enhance CaM kinase II activity in GH3 cells (Jefferson et al., 1991), raised the possibility that CaM kinase II might be involved in the transcriptional activation by TRH. To investigate this possisbility, I examined the ability of the CaM kinase II specific inhibitor 1-[N,O-Bis(5-isoquinolinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) (Hidaka et al., 1992) or its inactive analogue (KN-04) (Hidaka et al., 1992) to block TRH stimulation of PRL expression. I found that KN-62 effectively blocks the action of TRH, while its inactive analogue KN-04 does not (**Fig. 2.6**). To determine whether KN-62 is specific for CaM kinase under the conditions employed, I also examined whether KN-62 or KN-04 could block stimulation of PRL expression by TPA and FSK. I found that neither KN-62 nor its inactive analogue KN-04 inhibits TPA and FSK induction of PRL promoter activity (**Fig. 2.6**).

Since KN-62 can also inhibit the activity of two recently isolated members of the CaM kinase family, CaM kinase V (Mochizuki et al., 1993), and CaM kinase IV (also known as CaM kinase Gr) (Enslen et al., 1994), it is possible that any of these CaM kinases may be involved in the transcriptional action of TRH. Since we do not know whether CaM kinase V is expressed in GH3 cells, the possible involvement of this enzyme also remains to be investigated. Taken together, these data strongly suggest that

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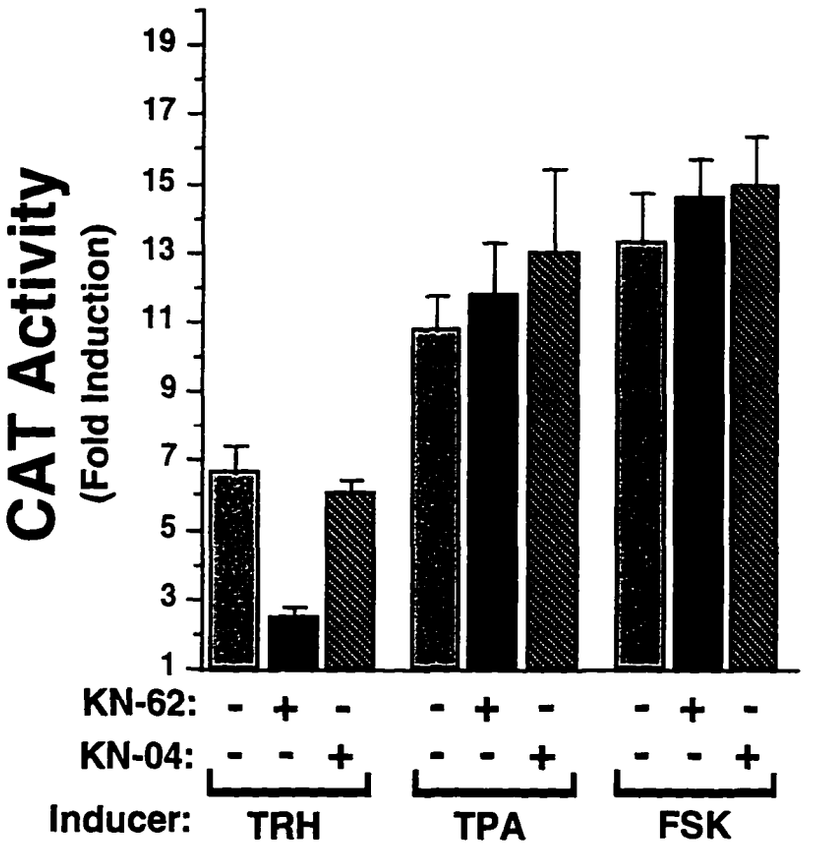

The CaM kinase II inhibitor KN-62 inhibits the action of TRH, while the inactive analogue (KN-04) does not. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubatedthree hours ± KN-62 (500 nM), the CaM KII inhibitor, or KN-04 (500 nM), the inactive analogue of KN-62 (20 μM), 18 hours ± TRH (100 nM) or TPA (100 nM) or FSK (1 μM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by TRH, TPA, or FSK observed with triplicate dishes.

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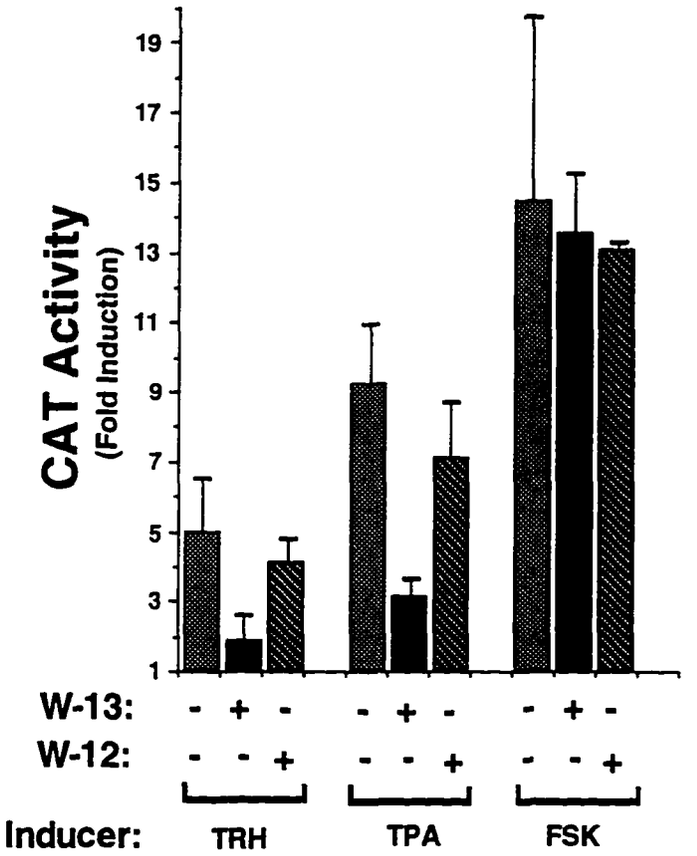

The calmodulin inhibitor W13 inhibits the action of TRH, while the inactive analogue (W12) does not. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated three hours± W13 (10 uM), the calmodulin inhibitor, or W12 (10um), the inactive analogue of W13, 18 hours ± TRH (100 nM) or TPA (100 nM) or FSK (1 μM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by TRH, TPA, or FSK observed with triplicate dishes.

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activity of a KN-62-sensitive CaM kinase is required for TRH action on the PRL promoter.

The involvement of CaM kinases in the TRH activation of the PRL promoter implies that one of its cofactors, calmodulin, should also be involved. As discussed earlier, Davis et al have shown that a relatively nonspecific calmodulin inhibitor, W-7 (Hidaka and Ishikawa, 1992), can inhibit TRH induced increase in PRL mRNA (Davis et al., 1991). To investigate whether calmodulin is involved in this pathway, I examined the ability of a more specific calmodulin inhibitor, N-(4-Aminobutyl)-5-chloro-2-naphthalene-sulfonamide (W-13) (Hidaka and Tanaka, 1983), and its inactive analogue (W-12) (Chafouleas, 1982) to block TRH action. As before, to verify the specificity of this inhibition, I investigated whether these compounds could also block TPA and FSK induced PRL expression. The results are shown in **Fig. 2.7**. I found that neither TRH, TPA nor FSK induction can be block by the inactive analogue W12. On the other hand, W13 inhibited both TRH and TPA stimulation of PRL-CAT expression. These results imply that calmodulin activity is required for TRH induction of the PRL gene. These results also indicate that TPA induced PRL expression may be dependent on calmodulin activity, but not CaM kinase activity. I have not investigated further these interesting observation.

5. Inhibitors of sarcoplasmic- or endoplasmic-reticulum calcium ATPase (SERCA ATPase) can block TRH induction of PRL promoter activity

TRH has been shown to activate PLC activity and increase the production of IP3 in GH3 cells (Gershengorn, 1982). By acting at an intracellular receptor (IP3 receptor) on the endoplasmic reticulum, IP3 stimulates the release of Ca^{2+} from intracellular stores (Gershengorn, 1982; Feris and Snyder, 1992). The difference between Ca^{2+}

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concentrations in the cytoplasm and the endoplasmic reticulum is established in part by a sarcoplasmic- or endoplasmic-reticulum calcium ATPase (SERCA ATPase) (Wuytak and Raeymaekers, 1992; Missiaen et al., 1991; Inesi et al., 1992; Brandl.et al., 1986). Thapsigargin, a sesquiterpene lactone tumor promoter isolated from the umbelliferous plant Thapsia garganica L. (Apiaceae) (Rasmussen et al., 1978), has been shown to induce the rapid emptying of the endoplasmic reticulum Ca^{2+} stores, by inhibiting the SERCA ATPase (Thastrup et al., 1990). The action of thapsigargin is specific to SERCA ATPase, since it does not inhibit the uptake of Ca^{2+} into plasma membrane vesicles (Thastrup et al., 1989). Thus, thapsigargin could be a useful tool for assessing the relationship between intracellular Ca^{2+} release and Ca^{2+} entry.

If IP3 is required for TRH stimulation of the PRL promoter, depleting the intracellular Ca^{2+} pool by thapsigargin, via a mechanism independent of both receptor activation and the generation of inositol phosphates, would abolish the TRH induction. As shown in **Figure 2.8**, 0.58 nM thapsigargin partially inhibits TRH induction, which is completely blocked by 2.34 nM thapsigargin. The observation of these effects at a low concentrations of thapsigargin suggests that these effects do not arise from non-specific cellular action of this inhibitor. This result supports a model in which IP3-induced Ca^{2+} release mediates TRH stimulation of the PRL promoter, suggesting that refilling of intracellular Ca^{2+} stores by SERCA ATPase is essential for TRH induction on PRL promoter.

It has been reported that application to cells of thapsigargin at concentrations up to 500 nM causes IP3-sensitive stores to be depleted without affecting the spontaneous Ca^{2+} oscillation that depends on the activity of L-type Ca^{2+} channels (Nelson et al., 1994). However, 20 μM thapsigargin blocked Ca^{2+} oscillation completely. To further verify the involvement of SERCA ATPase in the TRH transcriptional pathway, I used another SERCA ATPase inhibitor, cyclopiazonic acid (CPA), which is more specific

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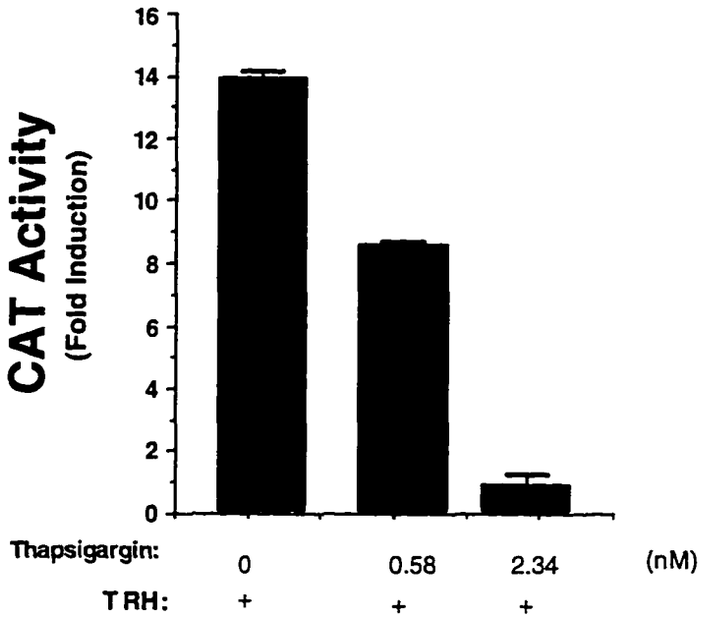

The Ca^{2+}-ATPase inhibitor thapsigargin blocks TRH stimulation of PRL promoter activity. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated three hours ± thapsigargin, then 18 hours ± TRH (100 nM), and assayed for CAT activity. Shown is TRH stimulation (fold ± SD) observed with duplicate dishes.

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and more reversible but less potent than thapsigargin (Nelson et al., 1994). I found that CPA yields a dose dependent inhibition of TRH action (**Fig. 2.9**). Since CPA depletes IP3-sensitive stores without inhibiting L-type channel activity (Nelson et al., 1994), this result further supports the hypothesis that the refilling of intracellular Ca^{2+} stores by SERCA ATPase is essential for TRH induction of PRL promoter activity.

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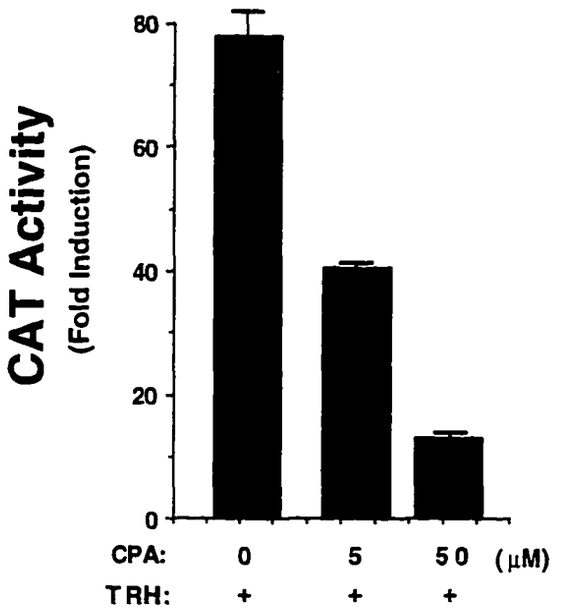

A more specific Ca^{2+}-ATPase inhibitor, cyclopiazonic acid (CPA), also blocks TRH stimulation of PRL promoter activity. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated three hours± cyclopiazonic acid (CPA) (5 or 50 μM), 18 hours ± TRH (100 nM), and assayed for CAT activity. Shown is TRH stimulation (fold ± SD) observed with triplicate dishes.

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DISCUSSION

Pertussis toxin (PTX) can uncouple several types of G proteins from their associated receptors by catalyzing the ADP ribosylation of a cysteine near the carboxy-terminus of the α subunit (Moss and Vaughn, 1988). Gi and Go have been shown to be PTX-sensitive, while Gq/11 and Gs are PTX-insensitive. PTX does not block TRH stimulation of PRL promoter activity (**Fig. 2.2**), implying the lack of involvement of a PTX-sensitive G protein. This result is consistent with the hypothesis that PTX-insensitive $G\alpha_{q/11}$ is the G protein subtype involved. However, since PTX reduces basal PRL expression (data not shown), a PTX-sensitive G protein(s) may also be important for PRL basal expression.

The L-type Ca^{2+} channel blocker nimodipine has been reported to block TRH induction of PRL promoter activity (Yan and Bancroft, 1991). Gi2, a PTX sensitive G protein, has been shown to be able to regulate L-type Ca^{2+} channels, and a constitutively active Gi2 can repress the rat PRL promoter (Lew et al., 1994). Since Gi is sensitive to PTX, the observation that PTX does not block TRH induction of the PRL promoter (**Fig. 2.2**) suggests that Gi2 does not mediate TRH stimulation of PRL promoter activity. It has been shown that dopamine repression of PRL promoter activity is mediated by one or more pertussis toxin-sensitive G proteins, and TRH does not prevent repression of PRL promoter activity induced by quinpirole, a D2 dopamine receptor agonist (Fischberg and Bancroft, 1995). These results suggest that TRH and dopamine work via different G proteins, consistent with the conclusion that the pertussis toxin-sensitive G protein, Gi2 , is not involved in the TRH pathway.

H-7 is an isoquinoline derivative, which inhibits PKC by directly competing with ATP to bind to the catalytic domain (Hidaka et al., 1992). Since the catalytic domain of protein kinase C shows homology to other protein kinases, H-7 does not exhibit a high

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degree of specificity for PKC (Hidaka et al., 1992). Calphostin C is a microbial compound isolated from Cladosporium cladosporioides. It is a highly potent and very specific inhibitor of PKC, inhibiting PKC by interacting with the common regulatory region present in all isoforms of this enzyme (Kobayashi et al., 1989). As shown in **Figures 2.3** and **2.4**, both H-7 and Calphostin C inhibit induction of the PRL promoter by TPA, while they have no effect on TRH induction of the PRL promoter activity. These results imply that PKC is not involved in TRH action on PRL transcription.

Martin et al have reported that TRH stimulation of PKC activation in GH3 cells is short lived, lasting less than 60 seconds (Martin et al., 1990). This suggests that the TRH-induced PKC activation may not last long enough effectively transduce a signal to the PRL gene activation pathway.

Gutierrez-Hartmann and coworkers have shown that constitutively active Ras or Raf can activate PRL promoter activity (Conrad and Gutierrez-Hartmann, 1992). This action of Ras is exerted via the Raf/MAP kinase pathway (Conrad and Gutierrez-Hartmann, 1992). It has been shown that Ets can be phosphorylated by MAP kinase (Macleod, 1992). Several putative Ets consensus-binding sites, 5' (A/C)GGAA-3', were found in the proximal PRL promoter (Macleod, 1992; Wasylyk et al., 1993). Overexpression of Ets-1 in GH3 cells increases both basal and Ras induction directed by the 3P element of the proximal PRL promoter (Howard et al, 1995). Although these findings raise the possibility that the Ras-Raf-MAP kinase-Ets pathway may be involved in TRH stimulation of the PRL promoter, my data showed that neither dominant negative Raf nor dominant negative Ras nor dominant negative Ets could block TRH induction.

The Raf-C4, a dominant-negative Raf mutant which contains only the Ras binding domain, has been shown to inhibit both basal level of expression from AP-1/Ets driven promoters as well as serum- and TPA-induced expression from the oncogene responsive

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element in the polyomavirus enhancer in NIH-3T3 cells (Bruder et al., 1992). As shown in Fig 2.5, the same Raf mutant did not block TRH induction in GH3 cells, raising the possibility that Raf activity is not required for TRH stimulation of PRL promoter. It was found that the same Raf mutant was able to block PRL promoter activity induced by constitutively activated Gq in GH3 cells (Jun and Bancroft, unpublished observation), suggesting that Raf-C4 acts as a dominant-negative inhibitor under the same conditions as those employed for the experiment depicted in Fig 2.5.

It has been shown that TRH induced phosphorylation of Raf-1 can be detected as early as 1 min after TRH addition, reaching a maximum at 2.5 min, and decreasing to control levels by 10 min (Ohmichi et al., 1994). Similarly, the maximum activation of MAP kinase was detected after a 5 min exposure to TRH and declined thereafter (Ohmichi et al., 1994). One explanation for the apparent lack of involvement of the Ras-Raf pathway in TRH action could be that the TRH-induced activation of Ras-Raf-MAP kinase-Ets pathway may be too short to effectively transduce a signal to the PRL gene. However, the possibility that MAP kinase acts only as an initiator of PRL transcription has not been ruled out yet. Thus, the possibility of involvement of the Ras-Raf-MAP kinase pathway in TRH stimulation of PRL promoter activity still needs to be further investigated. A direct way to examine this possibility would be to examine TRH-stimulated MAP kinase activity after cotransfecting with dominant negative Raf. If a dominant negative Raf were shown to block TRH-induced MAP kinase activity, the above conclusion would be further supported. However, since less than 10% of the cells were transfected in transient transfection, such studies may be hard to do. The other way to investigate this possibility would be to examine the ability of Raf-C4 to block induction of a target promoter of MAP kinase, such as JunB promoter (Coffer et al., 1994), under the same experimental conditions. If a dominant negative Raf blocked Ras-induced JunB promoter activity, but not TRH-induced PRL promoter activity, the

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above conclusion would be confirmed. It may be mentioned finally that since all dominant negative mutant Raf, Ras and Ets proteins I examined severely decreased basal expression of the PRL promoter, this pathway may be involved in basal expression of PRL promoter.

Experiments using the PKA and PKC inhibitor H-7 indicate that neither the PKA nor the PKC pathway mediates TRH action on PRL transcription. The results of experiments using dominant negative Ras, Raf or Ets suggest that TRH stimulation of the PRL promoter is mediated by a protein kinase other than MAP kinase. Since Ca^{2+} has been shown to be a mediator of TRH induction of the PRL promoter, a calcium-responsive protein kinase may be involved. It has been reported that CaM kinase II responds to both Ca^{2+} that enters the cell through voltage-sensitive Ca^{2+} channels and Ca^{2+} released from internal stores via the phosphotidylinositol pathway (Meyer, 1992). Since TRH both increases the level of intracellular Ca^{2+}, and significantly activates CaM kinase in intact cells (Jefferson et al., 1991), it seemed possible that CaM kinase II is involved in TRH action on PRL expression. Since KN-62 can competitively inhibit CaM kinase II activity both *in vitro* and *in vivo* by directly interacting with the calmodulin binding site of this enzyme (Hidaka, 1990; Tokumitsu, 1990), the observation that KN-62 blocks the TRH induction of PRL expression (**Fig. 2.6**) implies that a CaM kinase mediates the action of TRH on the PRL promoter. Since CaM kinase IV (also known as CaM kinase Gr), a recently discovered member of the CaM kinase family, is also strongly inhibited *in vitro* by KN-62, at an IC50 similar to what is required to inhibit CaM kinase II (Enslen et al., 1994), it is possible that either or both CaM kinase II or IV is involved in the action of TRH.

CaM kinase IV shares ~40 percent sequence identity with CaM kinase II isoforms, and both CaM kinase types share a similar structure organization (Nairn and Picciotto, 1994). In addition, the consensus phosphorylation site sequence of CaM kinase IV is

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the same as that of CaM kinase II (Nairn and Picciotto, 1994). However, CaM kinase II phosphorylates CREB at Ser^{133} only, whereas CaM kinase II phosphorylates CREB at both Ser^{133} and a second site, Ser^{142} (Sun et al., 1994). CaM kinase IV has been reported to be localized to the nucleus (Jesen et al., 1991), which would allow the kinase to have ready access to transcription factors when activated by Ca^{2+}. This implies that CaM kinase IV may mediate some of the effects of Ca^{2+} on transcription. Examination of the subcellular distribution of transiently expressed CaM kinase IV and CaM kinase II reveals that CaM IV enters the nucleus, while CaM kinase II does not (Mathews et al., 1994). However, Schulman and coworkers showed that CaM kinase II isoform δ_B is targeted to the nucleus in transfected 208F cells, a rat fibroblast cell line. The observation that the PRL promoter is responsive to the constitutively active form of CaM kinase II, but has little or no response to the constitutively active form of CaM kinase IV (Sun et al., 1994) supports the idea that it may be CaM kinase II that is involved in TRH action on PRL expression. Finally, although CaM V activity was also found to be inhibited by KN-62 (Mochizuki et al., 1993), the possibility of the involvement of this enzyme still needs investigation, particularly since to date there is no evidence that CaM kinase V is expressed in pituitary cells.

If regulation of prolactin gene expression involves activation of specific protein kinases, then it is likely that the actions of these kinases involve changes in the phosphorylation of specific transcription factors. Pit-1 is thus a likely candidate as a target for CaM kinase. Maurer and coworkers have shown that TRH can induce the transient phosphorylation of Pit-1, which does not persist as long as TRH induced transcriptional activation (Howard and Maurer, 1994). They also found that TRH-induced phosphorylation of Pit-1 can be blocked by chronic exposure to phorbol ester, conditions which do not block the ability of TRH to induce PRL transcription. Therefore my data and Maurer's together suggest that TRH-induced phosphorylation of

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Pit-1 is mediated by PKC, but that this phosphorylation of Pit-1 is not necessary for TRH-mediated enhancement of PRL gene transcription.

While Pit-1 phosphorylation may not appear to be required for TRH stimulation of the PRL promoter, Pit-1 has been shown to be a mediator of TRH action on PRL gene expression. Overexpression of a mutated Pit-1 protein, which contains the DNA-binding domain but lacks the major transactivation domain, was found to substantially block TRH induction of PRL promoter activity in GH3 cells (Yan and Bancroft, 1991). It may thus be a transcription factor which can interact with Pit-1 that is the direct substrate of the CaM kinase-mediated action of TRH on the PRL promoter.

The inhibition of the SERCA ATPase by thapsigargin is highly selective, as thapsigargin has little or no effect on the Ca^{2+}-ATPase of hepatocyte or erythrocyte plasma membrane or of cardiac or skeletal muscle sarcoplasmic reticulum (Thastrup et al., 1990). Previous studies by Hinkle and coworkers have demonstrated that treatment of GH3 cells for 30 min with 5 nM thapsigargin completely abolishes the TRH-induced spike in intracellular free Ca^{2+} (Nelson et al., 1994). Incubating GH3 cells with thapsigargin for 3 hours prior to the addition of TRH is sufficient for depleting the intracellular Ca^{2+} stores. Since thapsigargin has no effect on L-type channel activity at concentrations sufficient (lower than 500 nM) to completely block agonist-evoked Ca^{2+}-release (Nelson et al., 1994), it seems unlikely that effects of thapsigargin on L-type Ca^{2+} channel activity influenced my experiments (see **Fig 2.8**).

Cyclopiazonic acid (CPA), a SERCA ATPase inhibitor which is more specific and more reversible but less potent than thapsigargin (Nelson et al., 1994), also blocks TRH induction of PRL promoter activity (**Fig. 2.9**). CPA does not inhibit L-type channels, even causing a slight stimulation of $^{45}Ca^{2+}$ uptake through L-type channels (Nelson et al., 1994). Consistent with this observation, I found that CPA causes a small increase in the basal expression of the PRL gene. The observation that CPA depletes IP3-sensitive

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stores without inhibiting L-type channel activity (Nelson et al., 1994) provides further confirmation that the refilling of intracellular Ca^{2+} stores by SERCA ATPase is essential for TRH induction of the PRL promoter activity.

A tentative model for TRH regulation of PRL promoter based on my experiment results is shown in **Fig. 2.10**: Upon TRH binding, the TRH receptor activates PLC via a PTX-insensitive G protein. PLC then stimulates production of IP3, which causes the release of Ca^{2+} by binding to IP3 receptors on the ER membrane. TRH also induces Ca^{2+} influx via L-type Ca^{2+} channels. The increased intracellular Ca^{2+} then binds to calmodulin, causing the activation of a KN-62 sensitive Ca^{2+}/calmodulin-dependent protein kinase(s) which phosphorylates its gene-proximal substrate(s), most likely a protein other than Pit-1, possibly a Pit-1 binding protein. As a result, PRL gene transcription is stimulated.

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Cellular pathways of TRH action on the prolactin promoter

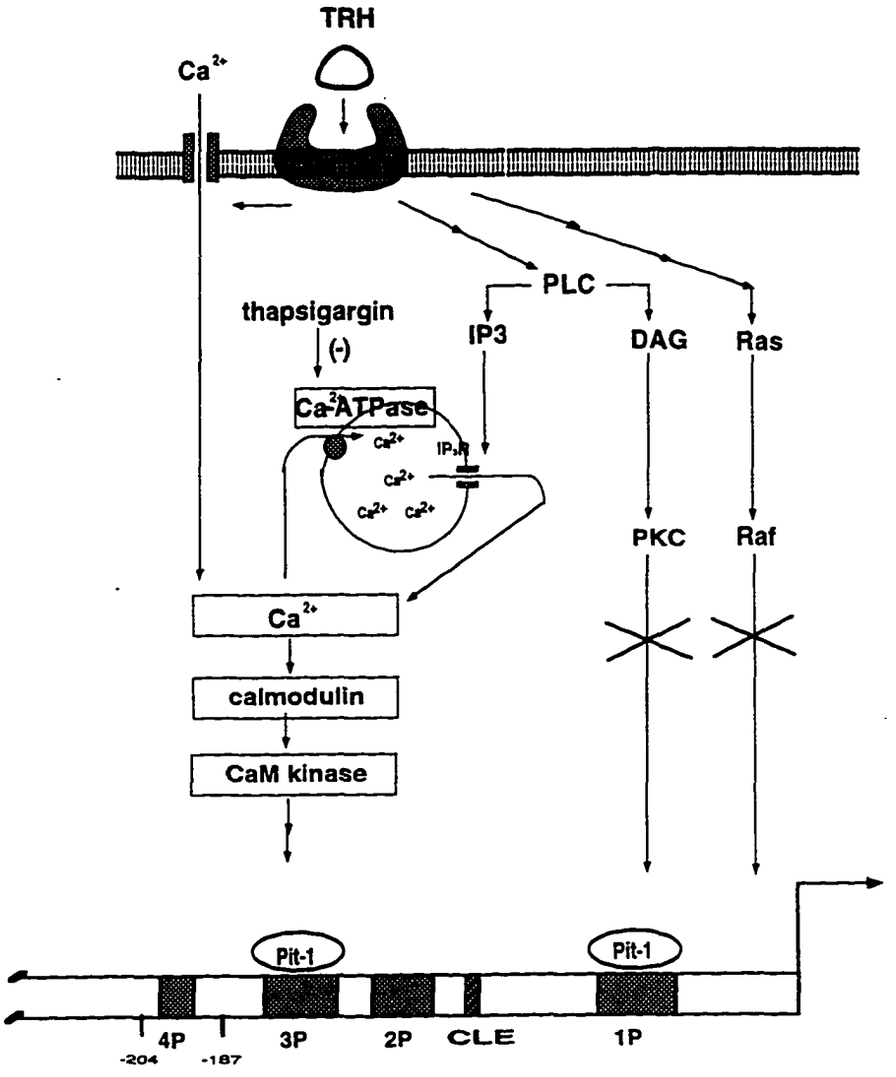

Figure 2.10

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Chapter III

TRH and PACAP Employ Independent Pathways to Regulate Prolactin Promoter Activity

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INTRODUCTION

1. Cellular actions of PACAP

It has been reported that PACAP receptors can be classified as type I or type II (Rawlings, 1994).. Type I receptors are highly specific for PACAP, whereas type II receptors bind both VIP and PACAP (Rawlings, 1994). For this reason, PACAP type II receptors are also called VIP receptors. Type I receptors have five splice variants: hip, hop1, hop2, hip-hop1, hip-hop2. Hip can stimulate only adenylate cyclase, while the other splice variants can activate both adenylate cyclase and phospholipase C (Spengler et al., 1993; Journot et al., 1995). Type II receptors correspond to either of two recently cloned receptors, VIP1 and VIP2 (Ishihara et al., 1992; Lutz et al., 1993), and activate almost exclusively adenylate cyclase (Rawlings, 1994). In the following, I will discuss further the transcriptional pathway activated by adenylate cyclase.

Activation of adenylate cyclase increases the cellular level of cAMP, resulting in the activation of PKA and phosphorylation of numerous protein substrates (Edelman et al., 1987). The phosphorylation of some of the cellular proteins alters the expression of target genes (Montminy et al., 1990). The study of cAMP responsive genes reveal a common cAMP response element (CRE), TGACGTCA (Montminy et al., 1986). Following phosphorylation by PKA, a 43 kDa protein termed CREB bound to this sequence is activated, thus, activating CRE-containing promoters (Montminy and Bilezikjian, 1987; Yamamoto et al., 1988). Multiple CRE-binding proteins have been identified by cDNA cloning, including CREB, ATFs, CREM and CREBP1 (Hai et al., 1989; Maekawa et al., 1989). These proteins all contain the "B-ZIP" structure, that is, a carboxyl-terminal basic region involved in sequence-specific DNA binding and a leucine zipper motif involved in protein-protein interaction. Many leucine zipper proteins have

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been shown to form either homodimers or heterodimers with other members of this class (Benbrook and Jones, 1990; Hai et al., 1991). For example, CREB form homodimers in solution (Dwarki et al., 1990), as well as form heterodimers with ATF-1 (Hurst et al., 1991) or CREM (Foulkes et al., 1991).

2. Pathway of action of PACAP on the PRL promoter

In Chapter II, I have described my studies of the TRH signal transduction pathway involved in regulating the PRL promoter. However, little is known about the molecular mechanisms of how PACAP mediates the induction of the PRL promoter. It has been reported that GH3 cells express transcripts for two PACAP receptors, the PACAP-R-hop1 isoform and VIP2 isoforms (Coleman et al., 1996). PACAP-R-hop1 can mediate PACAP stimulation of cellular levels of both cAMP and IP3 (Spengler et al., 1993), while VIP2 has been shown to selectively increase intracellular cAMP levels (Ishihara et al., 1992; Lutz et al., 1993). It is possible that PACAP may activate both adenylate cyclase and phospholipase C in GH3 cells (**Fig. 3.1**). Therefore, either or both of these signaling pathways might be involved in PACAP stimulation of the PRL promoter. Since TRH also activates phospholipase C in these cells (Gershengorn et al., 1984; Hsieh and Martin, 1992), the possibility that PACAP and TRH both utilize the same signaling pathway needs to be examined.

The PLC and PKA pathways are two major pathways possibly employed by PACAP to regulate the PRL promoter. In addition, the following evidence raises the possibility of the involvement of the Ras/Raf pathway. It has been reported that PACAP-38-induced activation of K^+ channels in Drosophila is mediated by coactivation of both the Ras/Raf and adenylate cyclase signaling pathways (Zhong, 1995). Therefore, it is worth examining whether PACAP induction of the PRL promoter acts via the Ras/Raf pathway.

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Possible cellular pathways for PACAP action on the prolactin promoter

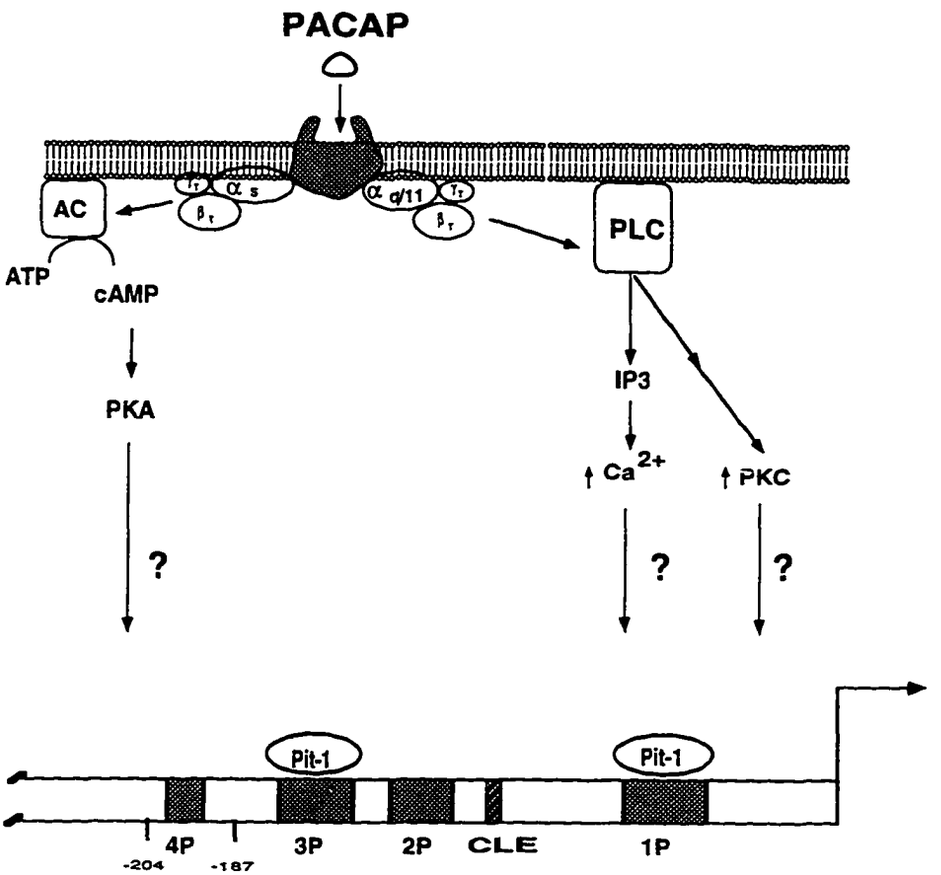

Figure 3.1

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In summary, PACAP could potentially activate several pathways in the GH3 cells, including the PLC/PKC pathway, the cAMP/PKA pathway, a Ras/Raf pathway, and a calmodulin/CaM kinase pathway (See **Fig. 3.1**). This raises the possibility that PACAP action may be mediated by either one or a combination of several pathways. In this chapter, I present evidence that PACAP stimulation of PRL promoter activity is exerted virtually entirely via a cAMP/protein kinase A (PKA)-mediated pathway. This pathway is thus different from the transcriptional pathway employed by TRH, which, as described in the previous chapter, requires CaM kinase activity.

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MATERIALS AND METHODS

Materials

PACAP-38 were purchased from Bachem California (Torrac, CA). Nifedipine and amiloride were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Conotoxin was kindly provided by Dr. Loggothetis (Department of Physiology, Mt. Sinai).

Sources of recombinant plasmids

RSV-KCREB, an expression vector for a dominant negative CREB whose Arg^{287} was changed to Leu, was kindly provided by Dr. Richard H. Goodman (Walton et al., 1992). It has been shown that KCREB fails to bind the CRE element and acts as a dominant CREB repressor, probably by forming a non-binding heterodimer with CREB or other member(s) of the CREB/ATF family (Walton et al., 1992). The PRL promoter-CAT reporter constructs (-204)PRL-CAT has been described previously (Lufkin et al., 1989). Plasmid pUC19 was purchased from New England Biolab (Beverly, MA).

Site-directed mutagenesis

(*CLE)(-204)PRL-CAT and (CLE*)(-204)PRL-CAT were prepared by the technique of site-specific mutagenesis without phenotypic selection as described by Kunkel and coworkers (Kunkel et al., 1987). The following is a brief description of how the mutations were made.

(1) Preparation of a Uracil-containing single-stranded DNA template.

In order to accomplish this, the PRL promoter segment from bp -204 to +38 was removed by Hind III from (-204)PRL-CAT and ligated into vector pUC19, which contains portions of pBR322 and M13mp19. The resulting plasmid, M13-PRLp was

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isolated following transformation into the *E.coli* dut⁻ung⁻strain (CJ236), which lacks both the enzyme dUTPase and uracil N-uracil N-glycosylase. Since dUTPase is responsible for degradation of dUTP, cells lacking this enzyme have elevated levels of dUTP, which effectively competes with TTP for incorporation into DNA. Since Uracil N-uracil N-glycosylase normally removes uracil from DNA, the cell lacking this enzyme stabilizes the uracil-containing DNA. Consequently, in strain CJ236, uracil is incorporated into DNA in place of thymine and is not removed (Tye and Lehman, 1977; Tye et al., 1978; Sagher and Strauss, 1983). Finally, the uracil-containing single-stranded M13-PRLp DNA is isolated and used as template for site-directed mutagenesis.

(2) Site-directed mutagenesis

In order to carry out site-directed mutagenesis, oligonucleotides necessary for making the mutants of interest were synthesized by the Brookdale Center Store (Department of Molecular Biology, Mt. Sinai Medical Center). The sequences of these oligonucleotides are as follows:

CB85: TCTAT TTCCC CGGTT AAGAT AGCC, corresponding to the antisense strand of rat PRL proximal promoter from -87 to -110, with the 4 bases in the left hand side of CLE changed from TGAC to CCGG.

CB86: CCAAT CATCT ATGGG GGTCA TTAAG, corresponding to the antisense strand of rat PRL proximal promoter from -80 to -104, with 4 bases from the right hand side of CLE changed from GGAA to CCCC.

Each of these oligonucleotides was hybridized to the uracil-containing template. Then, the hybrids were incubated with T4 DNA polymerase in the presence of dNTPs, including dTTP to synthesize the complementary strand. Thus, double-stranded DNA products were obtained and were transformed into the wild-type *E. Coli* strain JM109, which both removes the uracil from the template strand and further degrades it. Only the

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complementary strands containing mutations (specifically M13-*CLE-PRLp and M13-CLE*-PRLp) were left in JM109. Then, using M13-*CLE-PRLp and M13-CLE*-PRLp as templates, *E. Coli* synthesized the complementary strands, yielding double-stranded DNA. Finally, the double-stranded plasmids (M13-*CLE-PRLp and M13-CLE*-PRLp) were isolated and digested with Hind III, cutting out the mutated PRL promoter segments. Finally, these mutated PRL promoter segments were ligated back to the original CAT vector, called (*CLE)(-204)PRL-CAT and (CLE*)(-204)PRL-CAT, respectively. The mutations were confirmed by sequence analysis using a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio), as described in the manufacture's instructions.

Other procedures

As described in the previous chaper.

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RESULTS

1. PACAP action on the PRL promoter is not inhibited by pertussis toxin

Both TRH and PACAP have been shown to significantly increase the activity of the PRL proximal promoter (Yan and Bancroft, 1991; Coleman and Bancroft, 1993). Both TRHR and PACAP receptors are cell surface receptors belonging to G protein-coupled receptor family, which contains seven putative transmembrane domains. PACAP receptor subtype hop1 and TRH receptor have been shown to be expressed in pituitary GH3 cells (Gershengorn, 1993; Coleman et al., 1996). They appear to be coupled to pertusis toxin-insensitive G proteins of the Gq/11 family (Gershengorn, 1993; Journot et al., 1995). Therefore, it is possible that the actions of both PACAP and TRH are mediated through the same signaling pathway. My results in **Chapter II** imply that the effect of TRH on PRL gene expression is mediated via a PTX-insensitive G protein. Based on these observations, I initially examined whether the G protein that mediates PACAP induction is also PTX-insensitive. I found that PTX fails to block PACAP induction of (-187)PRL-CAT activity (**Fig 3.2**), implying that PACAP action is also mediated via a PTX-insensitive G protein.

2. The actions of TRH and PACAP on the PRL promoter are additive

The above data are consistent with the possibility that the actions of both TRH and PACAP are mediated through the same signaling pathway. To further investigate this possibility, I examined whether TRH and PACAP have an additive effect on the PRL promoter. If two hormones share identical signal transduction pathway for stimulation of gene expression, it would be expected that the maximal effect of one hormone would not be further increased by presence of the second hormone. As shown in **Fig. 3.3**, the maximum induction of PRL promoter by PACAP or TRH alone was achieved at

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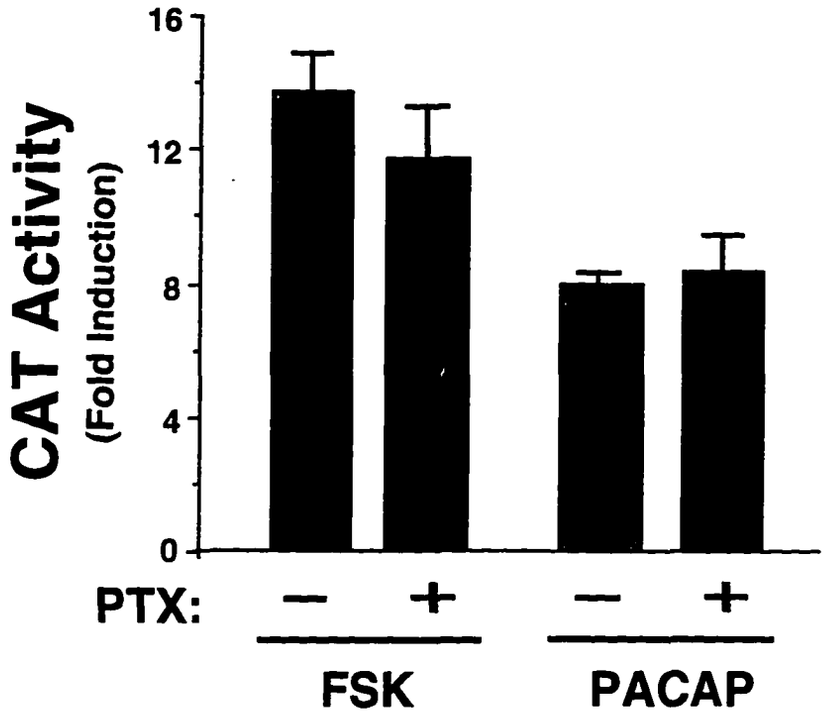

Pertussis toxin does not inhibit PACAP or FSK stimulation of PRL promoter activity. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated three hours ± pertussis toxin (200 ng/ml), 18 hours ± PACAP (100 nM) or FSK (1 μM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by PACAP or FSK observed with triplicate dishes.

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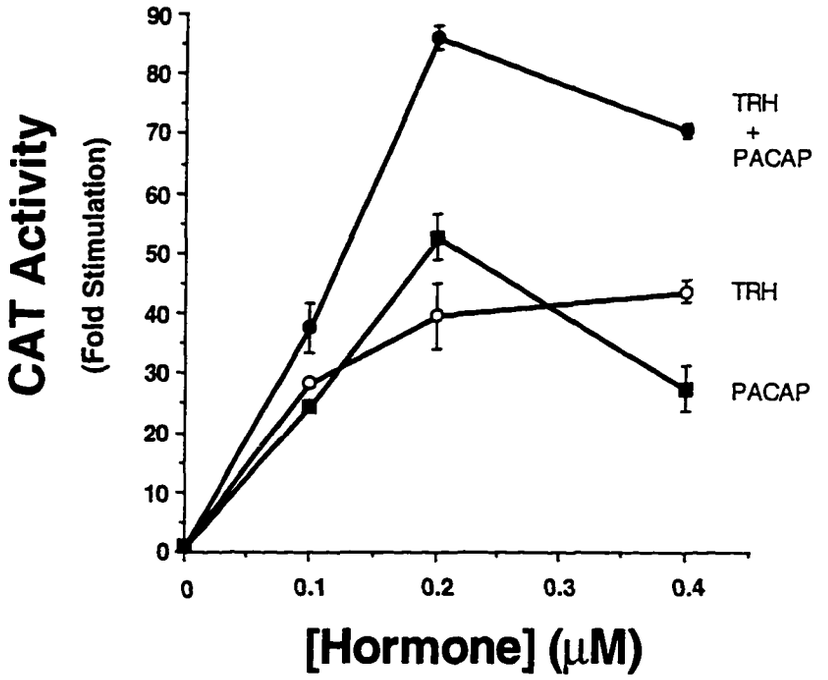

Stimulation by PACAP and TRH, alone or together, of PRL promoter activity. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated 18 hours wihout hormones, or with the indicated concentration of TRH and/or PACAP, and assayed for CAT activity. Shown is stimulation (fold ±SD) observed with triplicate dishes.

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200 nM. At this concentration, the effects of TRH and PACAP were approximately additive. This is consistent with the concept that PACAP and TRH stimulate PRL promoter via different pathways.

3. PACAP action on the PRL proximal promoter is not mediated by either a L-type channel blocker or the Raf pathway

The initial efforts to identify the PACAP signal transduction pathway were carried out by Dr. Daniel Coleman, a postdoctoral fellow in the Bancroft laboratory, and myself. I report below the results of my experiments.

Since PACAP has been shown to induce a rapid transient increase in the intracellular free calcium concentration in pituitary gonotropes and somatotropes (Canny et al., 1992), I tested whether L-type Ca^{2+} channel blocker nifedipine can block PACAP induction of the PRL promoter. As shown in **Fig. 3.4**, PACAP induces PRL promoter about 10 fold, and this induction was not decreased by the presence of nifedipine. However, I found that nifedipine does decrease basal expression of PRL promoter (data not shown). So, nifedipine, which was previously shown to block TRH induction (Yan and Bancroft, 1991), fails to block PACAP induction of the PRL promoter (**Fig. 3.4**). This finding further supports the hypothesis that PACAP and TRH stimulate the PRL promoter through different pathways.

Dr. Coleman did similar experiments with inhibitors of the PKA and PKC pathways. He found that both PACAP and FSK stimulation of PRL promoter activity were completely blocked by the PKA inhibitors RpcAMP and H89, but not by the PKC inhibitor staurosporin. In addition, PACAP fails to induce Ca^{2+} release from intracellular stores, although GH3 cells express PACAP type I receptor mRNA (Coleman et al., 1996). These results suggest that the action of PACAP on the transcriptional activity of the PRL promoter is mediated via cAMP/PKA. My

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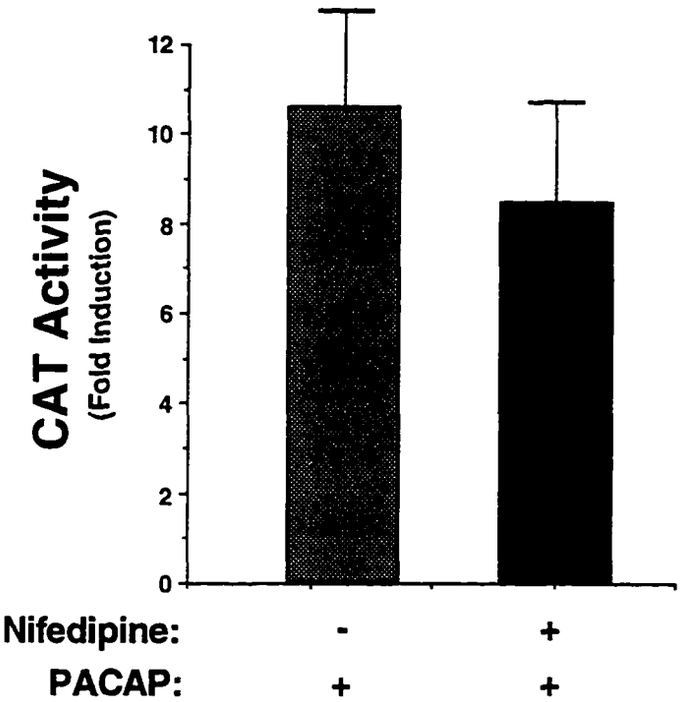

Nifedipine does not inhibit PACAP stimulation of PRL promoter activity. GH3 cells were transfected with (-187)PRL-CAT, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated three hours± nifedipine (5 μM), 18 hours ± PACAP (100 nM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by PACAP observed with triplicate dishes.

Figure 3.4

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observation described above (**Fig. 2.4**) that the specific PKC inhibitor calphostin C does not inhibit FSK induction of the PRL promoter supports these findings. Taken together, these results strongly suggest that PACAP induces the PRL promoter via the cAMP/PKA pathway, with PKC playing little or no role.

I also examined the possible involvement of the Ras-Raf-MAP kinase pathway in the transcriptional action of PACAP. I found that neither the dominant negative Raf kinase, Rafc4B, nor its partially inactivated mutant, Rafc4pm17B, blocks the action of either PACAP or FSK (**Fig. 3.5**). However, each of the mutated Raf proteins do decrease basal PRL promoter activaty (data not shown). Similar results were obtained with dominant negative mutants of Ras of the transcription factor Ets (data not shown). On the basis of these data, it appears that the Ras-Raf-MAP kinase signal transduction pathway is not involved in PACAP regulation of the PRL proximal promoter. Again, as discussed in Chapter II, further investigation is necessary to make a solid conclusion concerning this issue.

4. Gene-proximal events in PACAP and TRH action on the PRL proximal promoter.

4.1. The actions of PACAP and FSK on the PRL proximal promoter are mediated, at least in part, by a CREB-related protein, while the action of TRH is not. As described earlier, the PRL promoter contains a CRE-like (CLE) element, -99 TGACGGAA -92, which resembles the symmetric canonical cAMP response element TGACGTCA. Since PACAP appears to act via PKA (see above and Coleman, 1996 #2159], and CREB is a substrate of PKA, it is possible that PACAP action on the PRL promoter is mediated, at least in part, via the interaction of CREB (or a related protein) with the CLE element. In order to investigate this possibility, I

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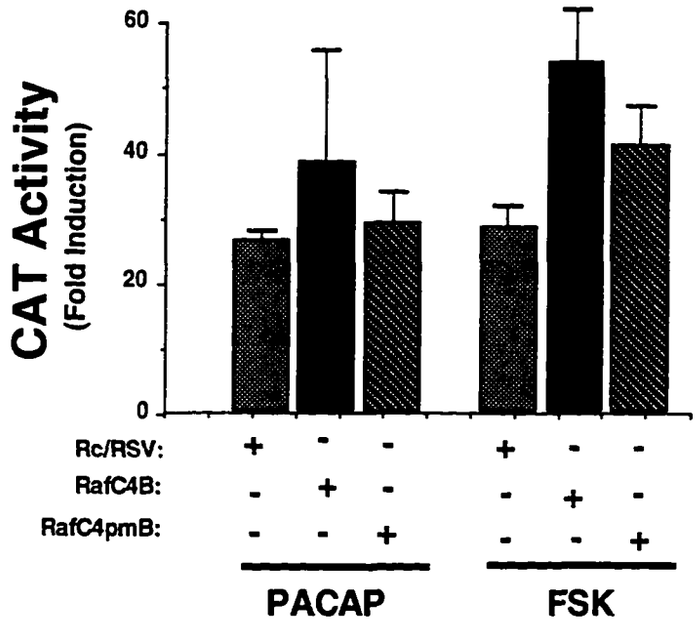

A dominant negative Raf mutant , RafC4B does not block PACAP stimulated PRL gene expression. GH3 cells were transfected with 10 μg (-187)PRL-CAT and 2.5 μg tk-GH plus 5 μg Rc/RSV or RSV-rafC4B (RafC4B) or RSV-rafC4 pm17B (RafC4pmB), divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated 18 hours ± PACAP (100 nM) or FSK (1 μM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by PACAP or FSK observed with triplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods.

Figure 3.5

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examined the ability of KCREB, a dominant negative form of CREB, to block PACAP and FSK induction. KCREB contains a mutation (Arg^{287} to Leu) in its DNA binding domain (Walton et al., 1992), but has an intact leucine zipper domain which is responsible for dimerization with other proteins of this family (**Fig. 3.6**). It has been shown that KCREB fails to bind to a CRE element and acts as a dominant CREB repressor, probably by forming a non-binding heterodimer with CREB or other members of the CREB/ATF family (Walton et al., 1992). Following cotransfection of (-187)PRL-CAT with increasing amounts of RSV-KCREB into GH3 cells, cells were incubated with either PACAP, FSK or TRH. As shown in **Fig. 3.7**, I found that both PACAP and FSK induction of the PRL proximal promoter were inhibited by KCREB in a dose-dependent manner at low inputs of the KCREB expression vector RSV-KCREB. PACAP induction of PRL promoter activity was reduced by 35% in the presence of 1.5 μg of RSV-KCREB. When the amount of RSV-KCREB transfected was increased, there was no further inhibition of PACAP action. Therefore, KCREB can only partially inhibit PACAP action on the PRL promoter. This implies that the action of PACAP on the PRL promoter is mediated, at least in part, by either CREB or another member of the CREB family capable of forming dimers with CREB; and in part by another type of protein. Similarly, FSK-induced PRL promoter activity was also partially inhibited by KCREB. By contrast, it is seen in **Fig. 3.7** that TRH stimulation of PRL promoter activity is not inhibited (and is even somewhat stimulated) at low concentrations of RSV-KCREB, but slightly inhibited at the highest concentration of RSV-KCREB studied (**Fig. 3.7**). These results thus further support the view that PACAP and TRH utilize different pathways to regulate PRL promoter activity. In addition, it should be noted that the basal activity of the PRL promoter is also inhibited by RSV-KCREB when 3 μg or less of RSV-KCREB is cotransfected into GH3 cells (see **Fig. 3.7**, lower panel). These data suggest that a CREB-like factor is involved not only in

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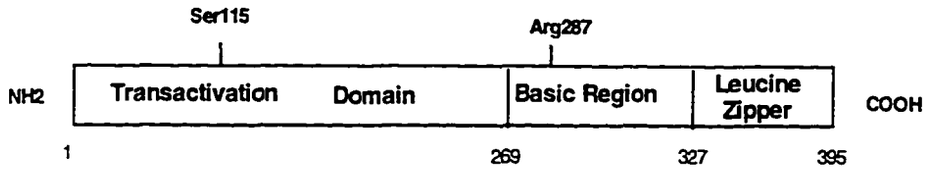
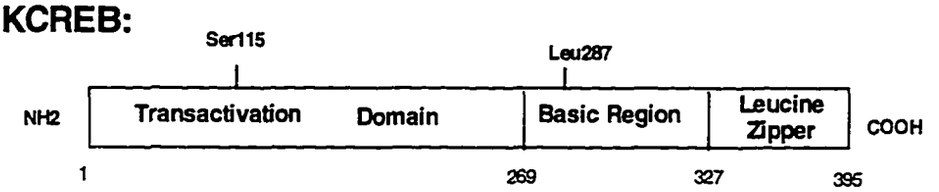
CREB:



KCREB:



Functional domains of CREB and KCREB, a dominant inhibitor of CREB

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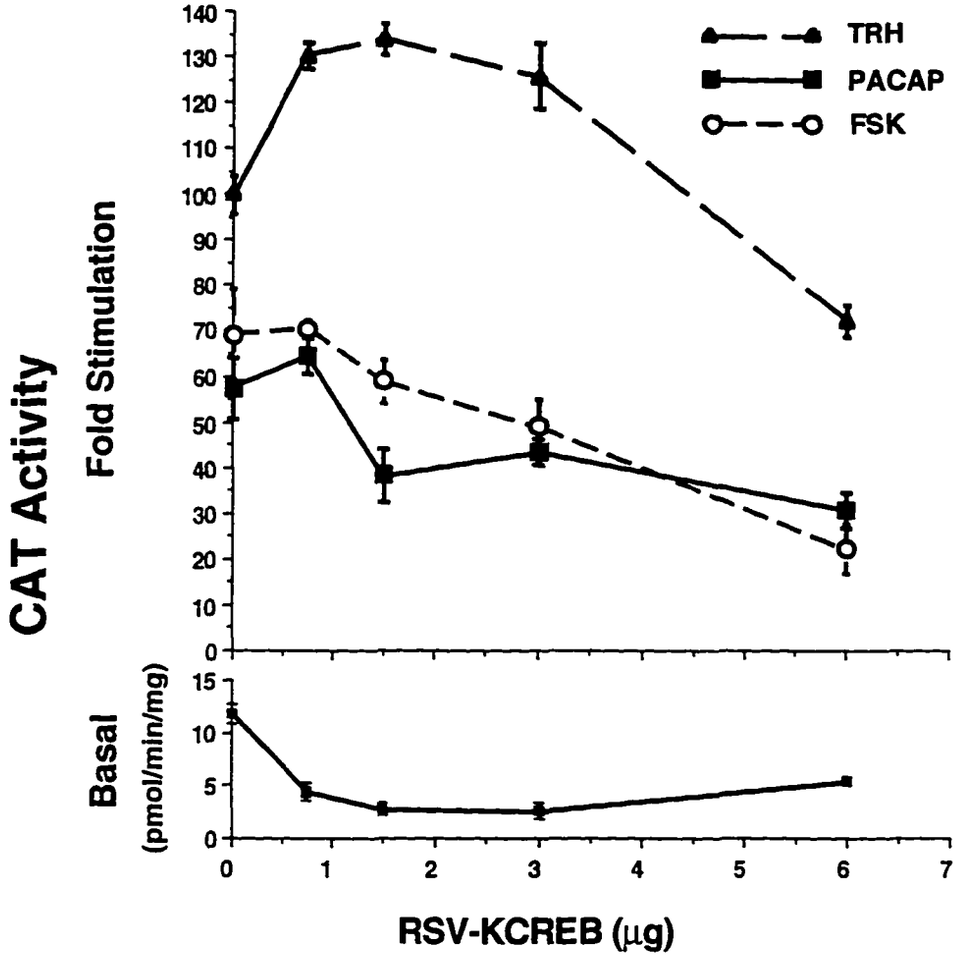

Stimulation of PRL promoter activity by either PACAP orFSK is inhibited by a dominant negative CREB mutant. GH3 cells were cotransfected with 5 μg (-187)PRL-CAT ($1.2X10^7$ cells) and 2.5 μg tk-GH plus the indicated quantities of RSV-KCREB, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated 18 hours ± PACAP (100 nM) or FSK (1 μM) or TRH (100nM), and assayed for CAT activity. Transfection efficiencies were normalized as described in Material and Methods. Data are presented either as fold stimulation (fold ± SD) of CAT activity, calculated following correction for hGH expression by the internal pTKGH control (upper panel), or as pmol acetylated chloramphenicol/min/mg (mean ±SD) secreted hGH assayed in control transfected cells (lower panel) observed with triplicate dishes.

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induction of PRL promoter activity by FSK or PACAP, but also the basal expression of the PRL gene.

4.2. The CLE element in the PRL promoter partially mediates PACAP and FSK induction, but not TRH induction. I examined whether the CLE element of the PRL promoter at position -99/-92 represents the target of the CREB-related protein implicated by the results presented in the proceeding section. I made two derivatives of (-204)PRL-CAT with mutations in the CLE by site-directed mutagenesis as described in the Materials and Methods. In (*CLE)(-204)PRL-CAT, the left-hand TGAC sequence of the CLE is changed to ccgg, while in (CLE*)(-204)PRL-CAT, the right-hand GGAA sequence of the CLE is changed to cccc (**Fig. 3.8**, upper panel). I found that both the (*CLE) and (CLE*) mutants decrease (-204)PRL-CAT basal expression (**Fig 3.8** legend). These data agree well with the observation that KCREB inhibits the basal expression of the PRL promoter (**Fig. 3.7** lower panel), and also with the previous finding that the CLE is responsible for basal expression of the PRL promoter (Keech et al., 1992; Liang et al., 1992; Jacob and Stanley, 1994). As is seen in **Fig. 3.8** that both the (*CLE) and (CLE*) decrease PACAP induction by about 50%. This finding agrees with the data which showed that KCREB only partially inhibits the induction of the PRL promoter. Similar results were observed for forskolin. In contrast, as shown in **Fig. 3.9**, neither mutations decreased TRH induction. Taken together, these results strongly suggest that the action of PACAP or FSK on the PRL promoter is mediated at least in part by the interaction of a CREB family member with the CLE.

In summary, the following observations imply that PACAP and TRH employ different pathways to regulate the PRL promoter: (1) The actions of TRH and PACAP on the PRL promoter are additive. (2) A dominant inhibitor of CREB, KCREB, partially

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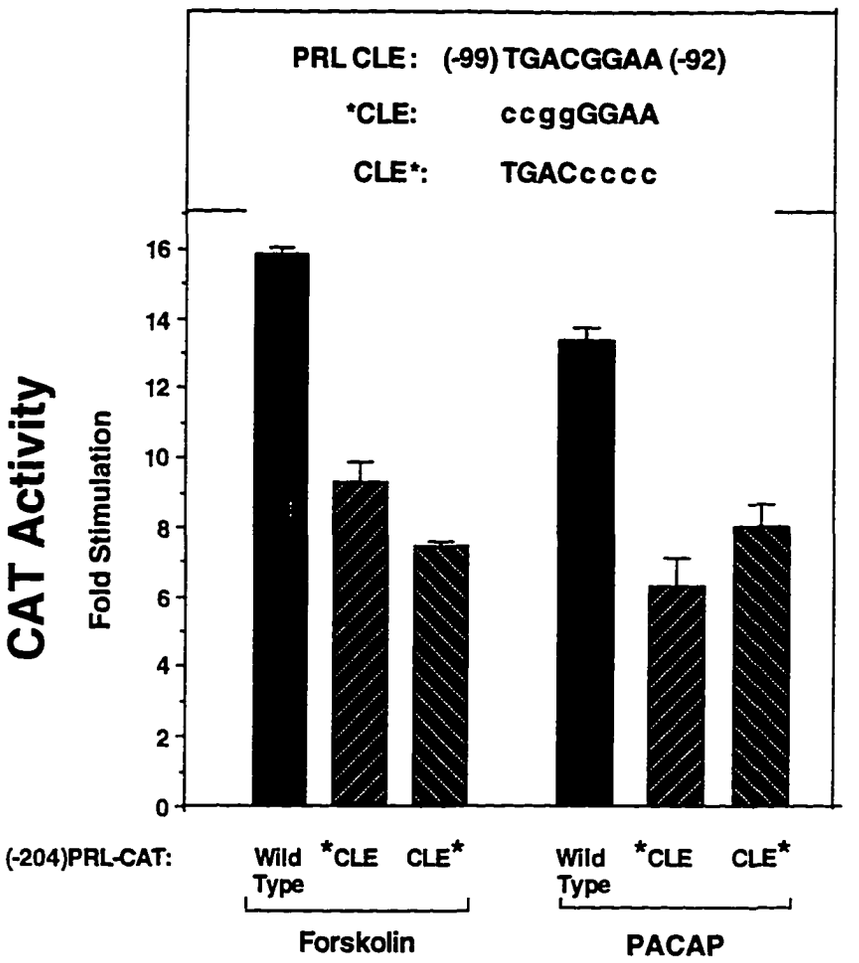

Mutations of the PRL promoter CLE sequence partially inhibit stimulation by either FSK or PACAP. GH3 cells were transfected with 10 µg (-204)PRL-CAT (**wild type**), or (*CLE)(-204)PRL-CAT (***CLE**) or (CLE*)(-204)PRL-CAT (**CLE***), plus 5 µg pTHGH, divided, and plated, all as described in Fig. 2.2. Following deinduction, the cells were incubated 18 hours ± PACAP (100 nM) or ± FSK, and assayed for CAT activity. Shown is stimulation (fold ± SD) by PACAP or FSK observed with triplicate dishes. The CAT activity, in pmol acetylated chloramphenicol/min/mg secreted hGH, observed in the control dishes incubated without additions, and transfected with either (-204)PRL-CAT, (*CLE)(-204)PRL-CAT or (CLE*)(-204)PRL-CAT, was respectively: 64.3±16.4; 25.3±4.5; 6.0±0.8.

Figure 3.8

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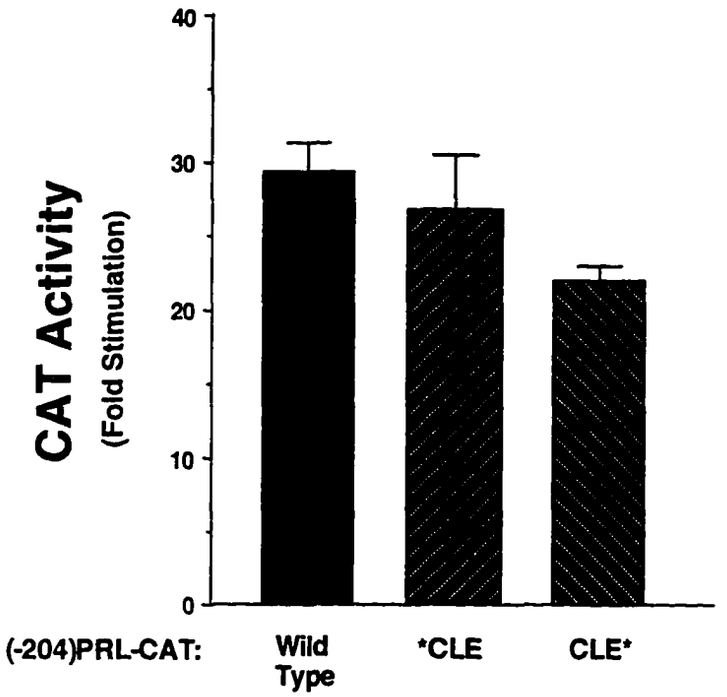

Mutations of the PRL promoter CLE have little effect on stimulation by TRH. GH3 cells were transfected, divided, and plated exactly as described in Fig. 3.9. Following deinduction, the cells were incubated 18 hours ± TRH (100 nM), and assayed for CAT activity. Shown is TRH stimulation (fold ± SD) observed with triplicate dishes.

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blocks the action of PACAP, but not of TRH. (3) Mutation of PRL promoter CLE element partially blocks the action of PACAP, but not of TRH.

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DISCUSSION

If TRH and PACAP employed identical signal transduction pathways for stimulation of expression of a gene, one would expect that the stimulation of expression of that gene by maximally effective concentrations of one hormone would prevent any further stimulation by the other hormone. My results showing that the effects of TRH and PACAP stimulate PRL promoter activity in an additive manner thus supports the idea that PACAP and TRH exert their effects through different pathways.

The observed additivity of TRH and PACAP effects on PRL gene expression could also be accounted for if receptor number was the limiting factor in the pathway for either ligand. However, preliminery results showed that TRH receptor is not the limiting step for the TRH induction on PRL proximal promoter in GH3 cells, since overexpression of the TRH receptor in GH3 cells fails to further enhance the TRH response (data not shown). Although we do not know whether PACAP receptors in GH3 cell are saturated, the further increases of PACAP maximum effect observed with TRH treatment makes this a less attractive explanation.

The maximum PACAP response is seen at a PACAP concentration of 200 nM. A decreased response was observed at high concentration of PACAP (400 nM); this is often observed at the high end of dose-response curves.

PACAP has been shown to induce a rapid transient increase in the intracellular free calcium concentration in PC12 cells (Deutsch and Sun, 1992) as well as in pituitary gonotropes and somatotropes (Canny et al., 1992), but not in GH3 cells (Coleman et al., 1996). This suggests that PACAP induces increases in intracellular Ca^{2+} in a cell type-specific manner. Further, I found that PACAP induction of the PRL promoter is not blocked by the L-type Ca^{2+} channel blocker nifedipine (**Fig. 3.4**). Since TRH induction has been shown to be blocked by the L-type Ca^{2+} channel blocker

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nimodipine, these results support the conclusion that PACAP and TRH work via different pathways.

I also examined the ability of other types of Ca^{2+} channel blockers to inhibit PACAP or TRH transcriptional pathways. I found that neither the T-type channel blocker amiloride nor the N-type channel blocker conotoxin blocks PACAP or TRH induction (data not shown). Therefore, the observation that, all three types of Ca^{2+} channel blockers tested (L-, N- and T-type) fail to inhibit PACAP induction of the PRL promoter in GH3 cells.

PKA pharmacological inhibitors as well as overexpression of the PKA regulatory subunit, Rab, have been shown to completely inhibit the induction of the PRL promoter by either PACAP or FSK (Coleman et al., 1996) and the effects of PACAP and FSK are completely non-additive (Coleman et al., 1996). Taken together, the results indicated that PACAP acts on the PRL promoter via a PKA-mediated pathway. Furthermore, since TRH receptor activation does not elevate cAMP levels in stably transfected cells expressing high levels of TRH receptors, including GHY, AtT-20, and Hela cells (Heinflink et al., 1994), the above findings lend further support to the idea that PACAP and TRH work via different signaling pathways.

TRH action on the PRL promoter has been shown to be mediated by Ca^{2+} (Yan and Bancroft, 1991). There is evidence that the effects of Ca^{2+} on the transcription of the c-fos gene involves the phosphorylation of CREB, the factor that has been shown to bind to CREs and mediate cAMP responsiveness (Sheng, 1990; Ziff, 1990). Ca^{2+} influx in PC12 cells leads to phosphorylation of CREB at Ser 133, the same residue that is phosphorylated by PKA (Sheng, 1990; Ziff, 1990). Since KCREB fails to block TRH induction of the PRL promoter (**Fig 3.9**), TRH action is likely to be mediated by Ca^{2+} via a mechanism different from the one involved in the regulation of the c-fos gene.

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The finding that KCREB partially inhibits FSK induction of the PRL proximal promoter differs from earlier studies by Liang and coworkers , in which a dominant CREB inhibitor did not block cAMP stimulation of the PRL promoter (Liang et al., 1992). However, the mechanism of inhibition by KCREB differs from that of the dominant negative CREB mutant used by Liang et al. Their mutant can bind DNA but not transactivate, acting as an inhibitor via direct competition for DNA binding. KCREB, on the other hand, can not bind to the CRE element, but represses cAMP stimulation probably by forming a non-binding heterodimer with CREB or another member of the CREB/ATF family (Walton et al., 1992). To verify this possibility, the ability of KCREB to reduce the binding of the CLE to the 100 kD protein which has been shown to bind to CLE of PRL promoter (Peers et al., 1992) should be addressed by gel shift experiments. It is also possible that the inhibition of PACAP/FSK induced PRL expression by KCREB is an indirect effect, due to reduced accumulation of a protein essential for PKA induction of PRL promoter. One candidate for this protein would be the 100 kD protein which can bind to the CLE of PRL promoter (Peers et al., 1992). Since the sequence of this protein is not known and antibody to this protein is not available, the influence of KCREB on the amount of this protein should be measured by far western assay, using radiolabeled CLE as probe. However, the finding that protein synthesis inhibitor cycloheximide fails to block FSK stimulation of PRL promoter in GH4C1 cells (Gilchrist and shull, 1993) does not support the concept of an indirect effect of KCREB.

The observation that CLE mutations partially block the PACAP and FSK response (**Fig. 3.8**) implies that the PACAP/FSK response of the PRL promoter may involve the CLE element. This conclusion is in agreement with the earlier observation that the same mutations in the CLE result in a modest loss in the transactivating ability of

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CREB-VP16 (Yan et al., 1994). My findings are also in agreement with the observation that synthetic multimers of CLE can mediate a cAMP response (Liang et al., 1992).

The observation that PACAP action on the PRL promoter was only partially inhibited by either RSV-KCREB or CLE mutations suggests that at least one additional region in the PRL promoter is also involved in the cAMP/PKA-mediated response to PACAP. It has been reported that the Pit-1 binding sites on the PRL promoter may be involved in the cAMP/PKA-mediated induction in GH3 cells (Keech et al., 1992; dEmden et al., 1992). The finding by Maurer and coworkers that disruption of Pit-1-binding sites can alter cAMP-mediated regulation of prolactin expression also supports this concept (Iverson et al., 1990). Finally, evidence presented in next chapter that a Pit-1 binding site 1P alone can directly mediate the FSK response (**Fig. 4.1**) confirms this hypothesis.

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Chapter IV

Protein Kinase A Regulates Prolactin Promoter Activity via Both Pit-1-Dependent and Pit-1-Independent Mechanisms

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INTRODUCTION

For many years, it has been known that the PRL promoter, inactive in nonpituitary cells (Nelson et al., 1988; Keech and Gutierrez-Hartmann, 1989), can be activated by cotransfection with a Pit-1 expression vector (Ingraham et al., 1988; Mangalam et al., 1989). The DNA elements which mediate the cAMP and TRH responsiveness of the PRL gene include Pit-1 binding sites found in the proximal PRL promoter (Day and Maurer, 1989). Mutagenesis of these Pit-1 binding sites can reduce the ability of cAMP to stimulate PRL expression (Iverson et al., 1990). Further, Peer et al. (1991) reported that the removal of Pit-1 binding sites within the human PRL proximal promoter region by 5' and 3' deletions correlates with decreased cAMP stimulation (Peers et al., 1991). These data suggest that Pit-1 binding sites are involved in the induction of PRL gene expression by cAMP. These data also raise the possibility that Pit-1 may play a role in PRL promoter regulation by cAMP.

Kapliloff et al. (Kapiloff et al., 1991) reported that Pit-1 is phosphorylated in rat pituitary cells (GC cells) at positions Ser^{115} in the transactivation domain, and Thr^{219} or Thr^{220} in the homeodomain, in response to either cAMP or phorbol ester treatment. Bacterially expressed Pit-1 can also be phosphorylated by PKA and PKC *in vitro*, but apparently not by CaM KII or casein kinase (Kapiloff et al., 1991). Following phosphorylation, Pit-1 exhibited slightly higher affinities for both the PRL promoter proximal site 3P and distal site 3D, and slightly lower affinities for the PRL 1P binding site and distal sites 1D, 2D and 4D (Kapiloff et al., 1991). These findings suggest that Pit-1 phosphorylation may play a role in Pit-1-mediated transcriptional events, including PKA-induced PRL gene expression.

Recently, Gellersen et al. (Gellersen et al., 1995) reported that full length (-8700 bp) human PRL promoter reporter constructs transfected into the human uterine sarcoma cell

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line SKUT-1B-20, which lacks both Pit-1 protein and mRNA, can be moderately stimulated by the PKA catalytic subunit. Interestingly, these cells do not use the decidual-specific upstream promoter of the hPRL gene, but instead transcribe the hPRL gene from the downstream pituitary-type transcription start site. Further studies showed that this effect of PKA on the full length PRL promoter was significantly enhanced by coexpression of Pit-1 (Gellersen et al., 1995).

In the studies described in this chapter, I have examined whether Pit-1 is required for PKA regulation of the PRL promoter. My results imply that PKA can regulate PRL promoter activity via both Pit-1-dependent and Pit-1-independent mechanisms, and that the Pit-1-dependent mechanism does not require Pit-1 phosphorylation.

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MATERIALS AND METHODS

Constructs

(-39)mMT-CAT and $(1P)^2$(-39)mMT-CAT were described previously. (Yan et al., 1991). Plasmids RSV-Pit-1 and RSV-Pit-1(A3) were constructed by Dr. Daniel Fischberg (Fischberg et al., 1994), using the corresponding constructs CMV-Pit-1 and CMV-Pit-1(A3), provided by Dr. M. G. Rosenfeld (Kapiloff et al., 1991).

Cell culture

AtT20 cells stably transfected with the TRH receptor (AtT20 (TRHR)) were kindly provided by Dr. M. Gershengorn (Cornell Medical School, New York, NY) (The presence of the TRH receptor was assumed not to influence either the FSK or the PMA stimulation experiments). AtT20 cells were maintained in monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% FCS (D-5), 100 mg/ml streptomycin, 100 U/ml penicillin at 37°C in humidified 5% CO_2 and 95% air. 235-1(P-) cells were obtained from Dr. Catanzaro (Cornell University Medical Center, New York, NY), and grown in monolayer culture in RPMI 1640 supplemented with 10% heat inactivated FCS, 100 mg/ml streptomycin, 100 U/ml penicillin. GH3 cells were cultured as described in Chapter II. Western blot analysis has shown that these cells express no detectable Pit-1 protein (B. Kloss and C. Bancroft unpublished observations.)

Calcium phosphate-mediated transfection

AtT20 cells were plated at $1X10^6$/60 mm dish in D-5 one day prior to transfection. Transfections were performed as described by Kingston et al. (Kingston, 1990). Briefly, 50 µl of 2.5 M $CaCl_2$ was mixed with 450 µl H_2O containing the DNA to be transfected. An equal volume of this DNA/$CaCl_2$ solution was added dropwise to 2XHBS solution (280 mM NaCl, 1.5 mM Na2HPO4, 50 mM Hepes, pH 7.05) and

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mixed by bubbling. Following vortexing for 5 seconds, the solution was left undisturbed for 20 min at room temperature, allowing for the formation of a precipitate. To each dish containing 5 ml of D-5, 500 µl of precipitate was slowly added in a dropwise manner. Following incubation for 6 hours, the medium was removed, and the cells were washed twice with 1X PBS and fed with fresh D-5 (5 ml).

Transfection by electroporation

GH3 cells or 235-1(P-) cells were centrifuged, and $5X10^6$ cells were resuspended in D-5 (0.5 ml) containing the DNA to be transfected. The cells received a single electrical pulse at 220 V from a total capacitance of 960 µF, using a Gene Pulser Transfection Apparatus (Bio-Rad, Hercules, California). Following electroporation, cells transfected with the same plasmid but from different cuvettes were pooled, and plated in serum-containing medium at $1X10^6$ cells per 35mm dish.

Site-directed mutagenesis

(1P*)(-204)PRL-CAT was made as described in Materials and Methods in Chapter II. The oligonucleotides CB87 (Synthesized by the Brookdale Center Store, Department of Molecular Biology, Mt. Sinai Medical Center) was used for making this mutant. The sequence of CB87 is as follows: TGCCTGATTATATATATAGGACTGAAGGTGTCGAA. This corresponds to the antisense strand of the rat PRL proximal promoter from positions -35 to -65, with four bases from the right-hand side of site 1P changed from TTCA to GGAC. This mutation disrupts the consensus Pit-1 binding site A/T TA A/T TCA. The mutation was confirmed by sequence analysis using a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio).

Other procedures

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RESULTS

1. The Pit-1 binding site, 1P, can confer forskolin responsiveness to a heterologous promoter

Since either KCREB or mutations in the CLE only partially block PACAP induction of the PRL promoter (see previous chapter), it is possible that other DNA elements may be involved in the action of PACAP. It has been suggested that Pit-1 binding sites in the PRL promoter are involved in the cAMP/PKA-mediated induction in GH3 cells (Keech et al., 1992; dEmden et al., 1992). To begin to investigate the involvement of Pit-1 in the PKA pathway, I examined the ability of the Pit-1 binding site, 1P, to confer a response to the adenylate cyclase activator forskolin (FSK). $(1P)^2$(-39)mMT-CAT contains two tandem 1P elements upstream of the cap site of the mouse metallothionein promoter (Yan and Bancroft, 1991). As shown in **Fig 4.1**, FSK yielded a 2.5-fold stimulation of $(1P)^2$(-39)mMT-CAT, while (-39)mMT-CAT did not respond to FSK stimulation. These data demonstrate that site 1P can direct a response to FSK. This effect is thus mediated by a nuclear protein(s) which can interact with 1P, most likely Pit-1 and/or another nuclear protein that can interact with Pit-1 and/or site 1P.

2. Phosphorylation of Pit-1 is not required for PKA mediated stimulation of PRL promoter activity

As discussed above, Pit-1 can be phosphorylated by protein kinase A, either in intact cells or *in vitro*, suggesting that Pit-1 is important in mediating the transcriptional regulation of the PRL gene. However, to date, there has been no direct evidence showing a functional requirement for Pit-1 phosphorylation. One way to elucidate the function of Pit-1 phosphorylation is to examine the ability of a PKA-unphosphorylatable mutant of Pit-1 to mediate FSK induction in Pit-1 deficient cells. The expression vectors

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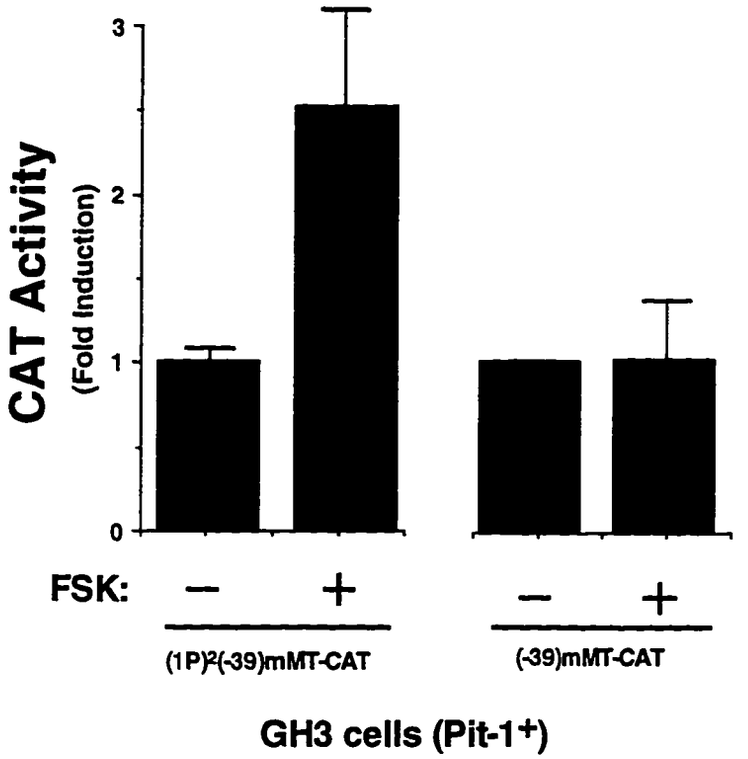

Pit-1 binding site 1P can mediate the transcriptional action of FSK. GH3 cells were cotransfected with 10 μg $(1P)^2$ mMt-CAT or -39mMT-CAT, 10 μg tk-GH per cuvette ($6X10^6$ cells) by electroporation. The transfected cells were divided, and plated as described in Fig. 2.2. Following deinduction, the cells were incubated for 18 hours ±FSK (1 μM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by FSK observed with triplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods.

Figure 4.1

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encoding either the PKA-unphosphorylatable Pit-1 or wild-type Pit-1 were kindly provided by Dr. M.G. Rosenfeld. The PKA-unphosphorylatable Pit-1, termed Pit-1(A3), is a Pit-1 mutant (Kapiloff et al., 1991) in which the known PKA phosphorylation sites (Ser^{115}, Thr^{219} and Thr^{220}) have been changed to alanines. The cells we used were AtT20 corticotroph cells which contain no Pit-1 (B. Kloss, unpublished results), and which are stably transfected with the TRH receptor [AtT20(TRHR)], supplied by Dr. M. Gershengorn (We assume the presence of the TRH recepter does not influence our results). Following transfection of these expression vectors (Pit-1 and Pit-1(A3)) into the AtT20(TRHR) cells, the cells were incubated ± FSK. As shown in **Fig 4.2**, FSK yields a 4-fold increase in PRL promoter activity in the presence of Pit-1. Surprisingly, in cells expressing Pit-1(A3) the transcriptional activation of the PRL promoter by FSK was comparable to that observed in cells expressing wild-type Pit-1. These data suggest that Pit-1 phosphorylation is not required for FSK stimulation of PRL gene expression.

3. The PRL proximal promoter responds to forskolin in pituitary cells that do not contain Pit-1

If Pit-1 phosphorylation is not required for the FSK induction of PRL gene expression, the next question would be whether Pit-1 itself is necessary for PKA activation of the rPRL promoter in lactotrophs. One way to answer this question is to investigate the PRL promoter response to FSK in a Pit-1-deficient lactotropic cell line. Brian Kloss in the Bancroft laboratory has found that the pituitary lactotropic cell line 235-1(p-) contains Pit-1 mRNA, but fails to synthesize any detectable Pit-1 protein (unpublished results). This makes 235-1(p-) a suitable cell line in which to carry out these studies.

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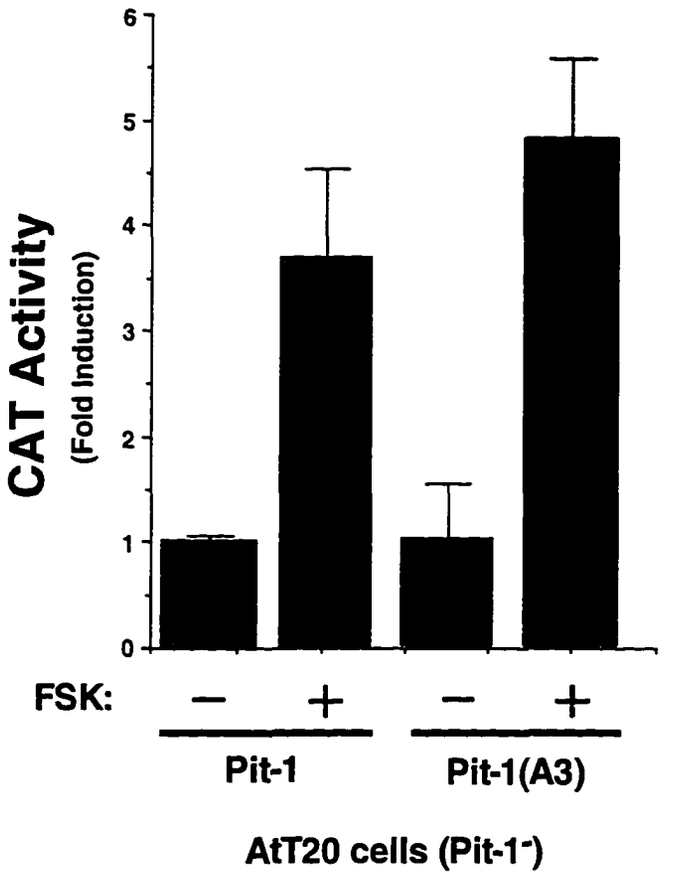

Phosphorylation of Pit-1 is not required for FSK action on the PRL promoter. AtT20 cells were cotransfected with 10 μg either RSV-Pit-1 or RSV-Pit-1 (A3), plus 10 μg tk-GH per dish ($1X10^6$ cells) by the method of calcium phosphate transfection. The transfected cells were divided and plated as described in Fig. 2.2. Following deinduction, the cells were incubated for 18 hours ±FSK (1 μM) and assayed for CAT activity. Shown is stimulation (fold ± SD) by FSK observed with triplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods.

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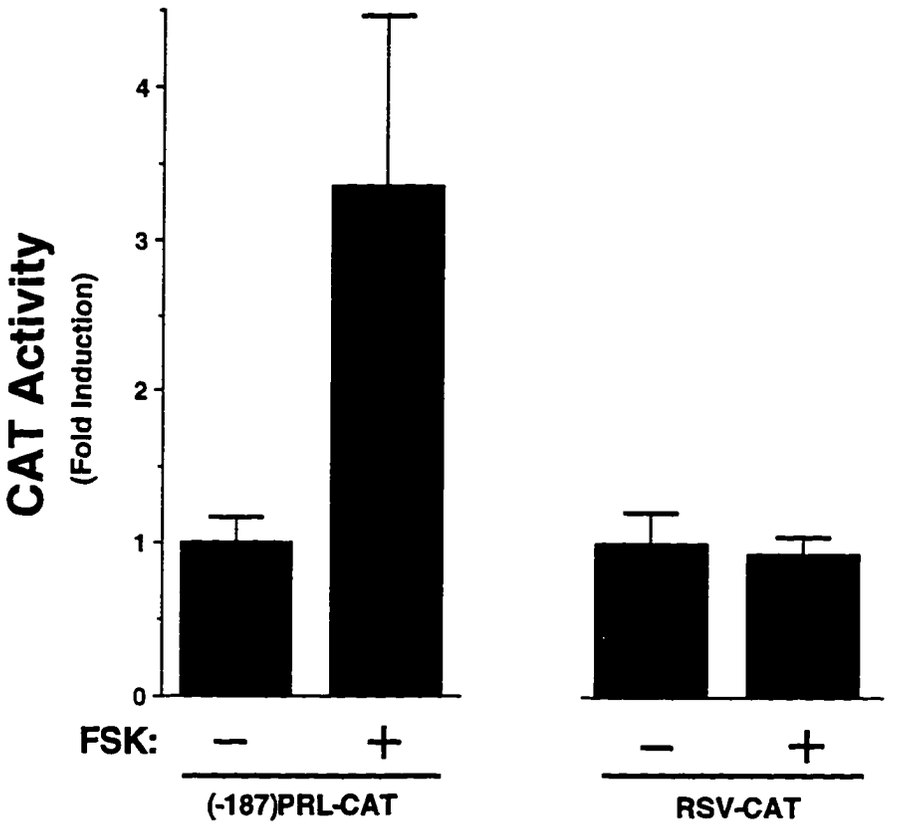

FSK can induce PRL promoter activity in pituitary lactotropic cells that lack Pit-1. GH3 cells were cotransfected with either 10 μg (-187)PRL-CAT or 2 μg RSV-CAT, plus 10 μg tk-GH per cuvette ($6X10^6$ cells) by electroporation. The transfected cells were divided, and plated as described in Fig. 2.2. Following deinduction, the cells were incubated for 18 hours ±FSK (1 μM) and assayed for CAT activity. Shown is stimulation (fold ± SD) by FSK observed with triplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods.

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FSK was observed to yield a 2-3 fold increase in expression of the (-187)PRL-CAT construct in 235-1(p-) cells (**Fig. 4.3**). However, FSK fails to stimulate RSV-CAT activity, indicating that the effect on PRL promoter activity is indeed specific. When (-1957)PRL-CAT, containing both the distal and proximal PRL regulatory regions, was used instead, FSK yielded a greater than 10-fold increase (data not shown). Cotransfecting these cells with RSV-PKA produced results similar to those obtained using FSK (data not shown). These results imply that FSK can stimulate PRL promoter activity in a Pit-1-independent manner. Thus the PRL promoter contains at least one Pit-1-independent PKA response element.

4. Mutations in the CLE significantly inhibit FSK induction of the PRL proximal promoter in pituitary lactotropic cells that do not contain Pit-1

To explore further the mechanism of FSK action in 235-1(p-) cells, I compared the FSK response of (-204)PRL-CAT with that of the (*CLE)(-204)PRL-CAT and (CLE*)(-204)PRL-CAT constructs described previously (see **Fig. 3.8**). As shown in **Fig. 4.4**, FSK stimulation of the constructs containing either CLE mutation was significantly lower than that observed with wild-type (-204)PRL-CAT, suggesting the invovement of the CLE in the Pit-1-independent stimulation by FSK. As a control, FSK stimulation of (1P*)(-204)PRL-CAT, which contains a mutation in the right-hand side of 1P, the high affinity Pit-1 binding site, was examined (**Fig. 4.4**). This construct responded to FSK as effectively as the wild-type PRL promoter in these Pit-1 deficient cells. It is thus likely that the CLE is involved in the Pit-1-independent stimulation of the PRL promoter by FSK.

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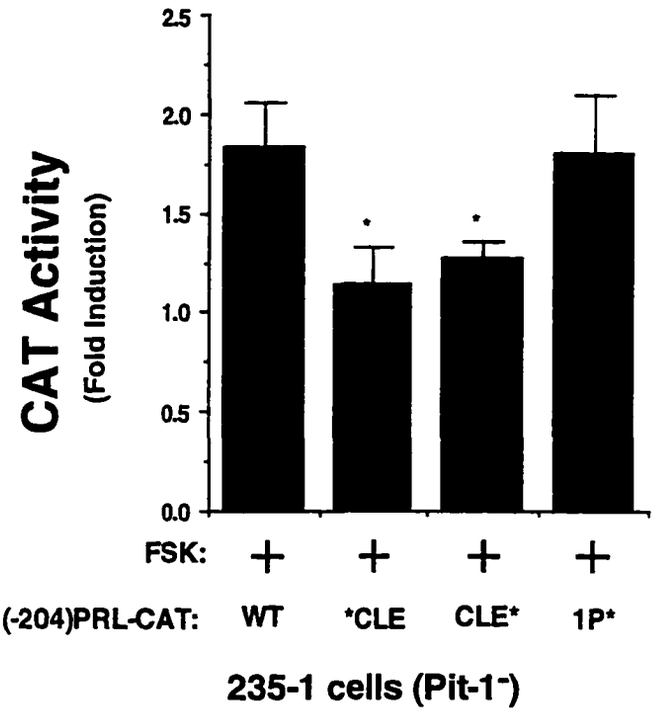

Mutations in the CLE slightly but significantly inhibit FSK action.
GH3 cells were cotransfected with 10 μg of either (-204)PRL-CAT, (*CLE)(-204)PRL-CAT, (CLE*)(-204)PRL-CAT, or (1P*)(-204)PRL-CAT, along with 10 μg tk-GH per cuvette ($6X10^6$ cells) by electroporation. The transfected cells were divided, and plated as described in Fig. 2.2. Following deinduction, the cells were incubated for 18 hours ±FSK (1 μM) and assayed for CAT activity. Shown is stimulation (mean fold ± SE) by FSK from three independent experiments with triplicate or duplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods. * Indicates $p < 0.05$ versus WT by t-test.

Figure 4.4

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5. Pit-1 is required for maximum stimulation by FSK of the PRL promoter

Since the FSK stimulation of PRL promoter activity in 235-1(P-) cells is much less pronounced than the stimulation in GH3 cells (compare **Fig. 4.3** with **Figs. 2.2** and **2.3**), it is possible that the relatively low level of the FSK response of the PRL promoter in 235-1(p-) cells may be due, at least in part, to the lack of Pit-1. To further study the possible involvement of Pit-1 in the FSK induction of the PRL promoter, 235-1(p-) cells were exposed to FSK following cotransfection with either the control Rc/RSV vector or RSV-Pit-1. As shown in **Fig. 4.5**, transfection of RSV-Pit-1 into the Pit-1-deficient cell line 235-1(P-) slight increased basal activity of the PRL promoter, and dramatically increased the FSK response of this promoter. The effect of FSK and RSV-Pit-1 on the PRL proximal promoter thus appears to be synergistic. These data imply that FSK action on the PRL proximal promoter is also mediated by a Pit-1-dependent pathway(s).

6. A Pit-1 phosphorylation mutant is as effective as wild type Pit-1 in acting synergistically with FSK to stimulate PRL promoter activity.

I have shown previously (**Fig. 4.2**) that Pit-1 phosphorylation is not required for FSK stimulation of PRL gene expression. To examine whether Pit-1 phosphorylation is required for the synergistic effect of FSK and Pit-1 described above, I transfected expression vectors for either Pit-1 or its phosphorylation mutant Pit-1(A3) into 235-1(p-) cells, and examined the effect of FSK. As shown in **Fig. 4.6**, FSK and Pit-1 again exhibited a synergistic effect, yielding together a 25-fold stimulation. Pit-1(A3) yielded a similar synergistic effect with FSK. These results suggest that Pit-1 phosphorylation is not required for the synergistic effects of FSK and Pit-1 on PRL promoter activity.

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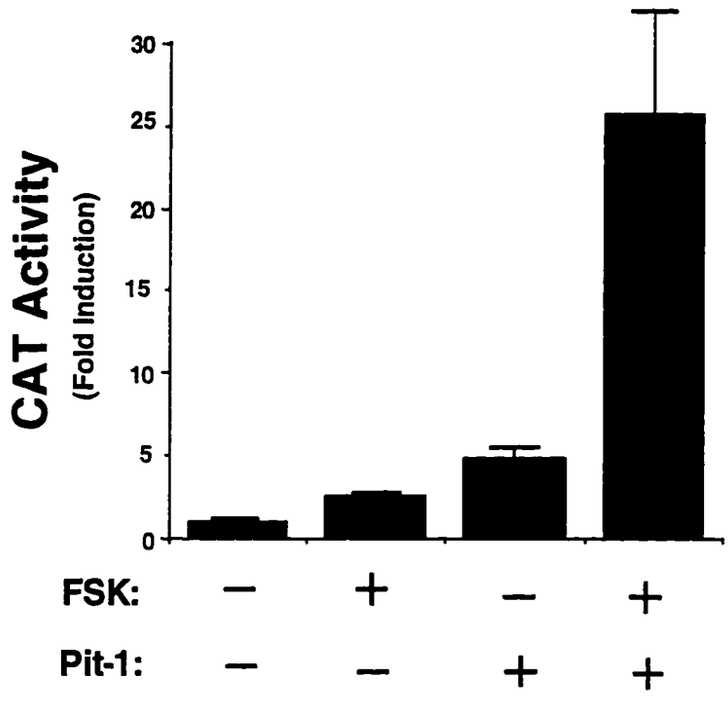

FSK and Pit-1 exhibit a synergistic activation of PRL promoter activity. 235-1(p-) cells were transfected with 10 μg (-187)PRL-CAT plus 5 μg Tk-GH and 10 μg RSV-Pit1 or/and Rc/RSV per cuvettee ($6X10^6$ cells) by electroporation. The transfected cells were divided, and plated as described in Fig. 2.2. Following deinduction, the cells were incubated for 18 hours ±FSK (1 μM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by FSK observed with triplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods.

Fig. 4.5

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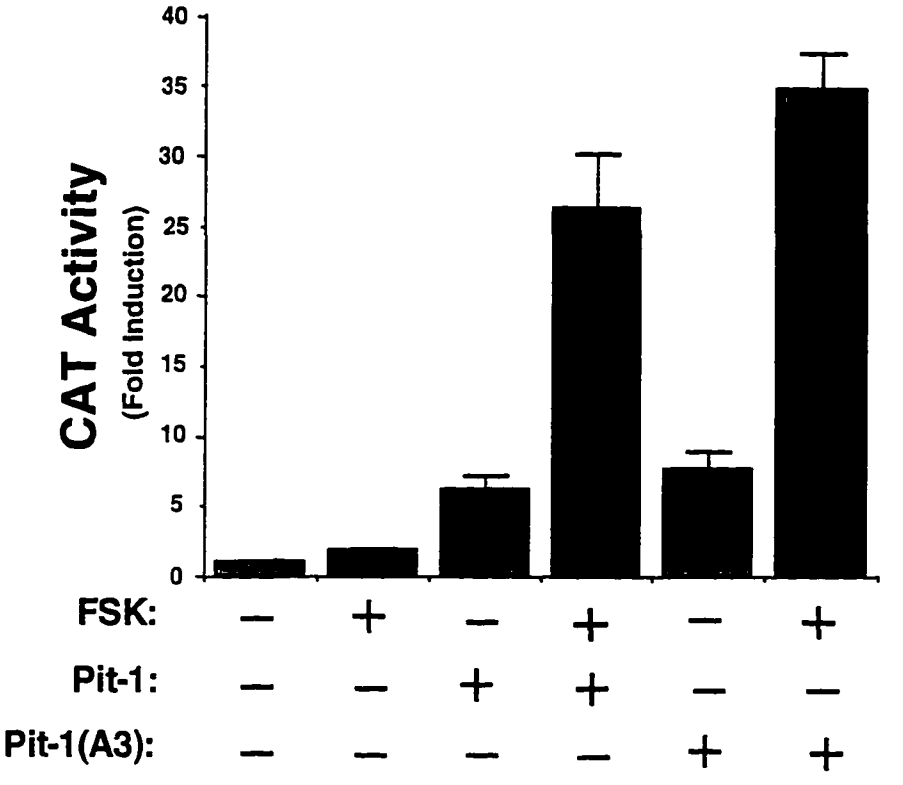

A Pit-1 phosphorylation mutant is as effective as wild type Pit-1 in yielding a synergistic action with FSK in stimulating PRL promoter activity. 235-1(p-) cells were transfected with 10 µg (-187)PRL-CAT plus 5 µg Tk-GH and 5 µg RSV-Pit1 or RSV-Pit1(A3) or Rc/RSV per cuvette ($6X10^6$ cells) by electroporation (320 V, 960 µF). The transfected cells were divided and plated as described in Fig. 2.2. Following deinduction, the cells were incubated for 18 hours ±FSK (1 µM), and assayed for CAT activity. Shown is stimulation (fold ± SD) by FSK observed with triplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods.

Fig. 4.6

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DISCUSSION

As shown in **Fig. 4.1**, the Pit-1 binding site 1P of the rat PRL promoter can direct a response to FSK. This observation supports the earlier finding by Maurer that disruption of Pit-1-binding sites can alter cAMP-mediated regulation of PRL promoter activity (Iverson et al., 1990). These data are also consistent with the finding that site 1P of the rat PRL promoter alone can also direct a response to Gαs (Tian et al., 1994), which exerts its cellular action via an increase in cAMP levels (Gaiddon et al., 1995). The Pit-1 binding sites in the human PRL promoter may also be involved in the action of FSK, since Peers et al reported that when five copies of site 1P or three copies of site 2P from the human PRL promoter are inserted in front of the thymidine kinase promoter, a significant induction by cAMP is observed in GC cells (Peers et al., 1991).

As shown in **Fig. 4.3**, FSK can induce PRL promoter activity in lactotropic cells in the absence of Pit-1. This Pit-1-independent action of FSK can be significantly blocked by mutation of the CLE, but not of site 1P (**Fig. 4.4**), suggesting that this action may be mediated by the CLE. This conclusion agrees with an earlier finding that a reporter construct containing eight copies of the CLE cloned in front of the thymidine kinase promoter responds to cAMP in rat-1 cells which lack Pit-1 (Liang et al., 1992).

Previous work in the Bancroft laboratory showed that CREB-VP16 strongly and specifically activates expression of (-187)PRL-CAT (Yan et al., 1994). In JEG-3 cells, a cell line derived from a human placenta choriocarcinoma, and in GC cells, a rat pituitary cell line, a reportor construct containing two copies of a CLE cloned in front of the thymidine kinase promoter responded to cAMP induction (Peers et al., 1991). Each of these observations lends further support to the hypothesis that a CREB-like factor can stimulate PRL expression in the absence of a Pit-1 binding site.

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Since the response to FSK observed in a Pit-1-deficient cell line (235-1) is much less than that seen in Pit-1-containing cells (GH3) (**Fig. 4.3** vs **Fig. 2.2**) and the effects of Pit-1 and FSK are synergistic (**Fig. 4.5**), it appears that Pit-1 does play an important role in the cAMP regulation of the rPRL promoter. This conclusion is supported by several previous observations. First, removal of the Pit-1 binding sites one by one from the 5' end of the PRL promoter gradually reduces cAMP stimulation (Peers et al., 1991). Second, several copies of either site 1P or 2P, when placed in front of the thymidine kinase promoter, confer cAMP responsiveness (Peers et al., 1991). Third, mutations within each Pit-1 binding site reduces cAMP stimulation of rat PRL promoter activity (Iverson et al., 1990). Finally, it has been shown that Pit-1 is required during pre-initiation complex assembly to activate transcription of the PRL gene *in vitro*, whereas a preformed class II preinitiation complex is refractory to Pit-1 influence (Sharp, 1995), suggesting that Pit-1 activates transcription by influencing the formation of type II pre-initiation complexes on target promoters.

Taken together, these data strongly suggest that Pit-1, along with the DNA elements which are known to interact with Pit-1, are important for FSK responsiveness. However, the finding that a Pit-1 phosphorylation mutant is as effective as wild type Pit-1 in mediating FSK induction (**Fig. 4.2**) raises the possibility that Pit-1 may not be the direct substrate of PKA. Instead, a protein that interacts with Pit-1 (Labeled X in **Fig. 4.7**) may be the direct protein substrate for PKA action mediated by a Pit-1 binding site (**Fig. 4.7**). Furthermore, these data implicate two types of cis-acting elements, the Pit-1 binding site and the CLE, in FSK action on the PRL promoter. Similar results were obtained in studies of the human growth hormone promoter (Shepard et al., 1994). In those studies it was found that PKA stimulation of human growth hormone gene expression requires both CRE motifs and a Pit-1 binding site. Unlike my result with the rat PRL promoter, however, overexpression of Pit-1 in

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cotransfection studies increased only the basal level of hGH promoter expression, while not affecting the levels of FSK induction (Shepard et al., 1994). Previous work in the Bancroft laboratory showed that a constitutively active form of CREB, CREB-VP16, transactivates the PRL promoter synergistically with Pit-1 in non-pituitary cells (Yan et al., 1994), consistent with my finding that FSK and Pit-1 exibit a synergistic activation of PRL promoter activity (**Fig. 4.5**).

In summary, a tentative model for PKA-mediated regulation of PRL promoter activity is as follows (see **Fig. 4.7**): Both the Pit-1 binding site 1P and the CLE of the PRL promoter are involved in FSK stimulation. Thus, PKA acts via both Pit-1-dependent and Pit-1-independent mechanisms to stimulate PRL promoter activity. A CREB-like protein(s), interacting with the CLE, may act coordinately with Pit-1 and/or a Pit-1 interacting protein(s) to mediate the cAMP-dependent stimulation of PRL gene transcription. Once the protein that interacts functionally with the CLE has been identified, it will be interesting to determine whether it can exhibit a physical interaction with Pit-1.

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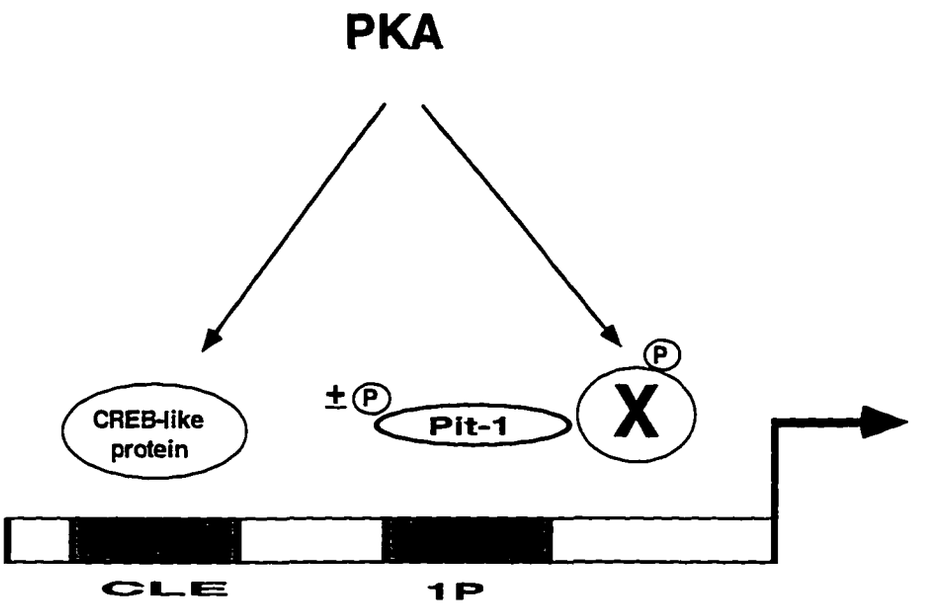

Model: mechanism of PKA mediated regulation of the PRL promoter activity.

Figure 4.7

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Chapter V

Preliminary Investigations of TRH and PACAP Action on Another Promoter Containing a Pit-1 Binding Site: the Pit-1 Promoter

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INTRODUCTION

Analysis of the relative contributions to PRL promoter activity of individual Pit-1 binding sites found in the proximal PRL promoter region, which contains four such sites, has been complicated because of functional redundancy of these elements (Iverson et al., 1990; Keech et al., 1992). For example, point mutations in any single Pit-1 binding site does not alter the stimulation obtained with TRH (Iverson et al., 1990). My preliminary observation that the mutation of the Pit-1 binding site 1P in the PRL promoter fails to block TRH induction (data not shown) also supports the above conclusion. To better understand the role of a Pit-1 binding site in mediating TRH and PACAP stimulation of a target gene, I used the proximal Pit-1 promoter, which contains a single Pit-1-binding site (see **Fig. 5.1A** below) as a model.

Analysis of the Pit-1 gene region indicates the presence of two autoregulatory elements, a positive element and a negative element, which are homologous to the Pit-1-binding sites found in both the GH and PRL promoters (McCormick et al., 1990; Chen et al., 1990). The positive element is located in the Pit-1 promoter between position -70 and -38 (McCormick et al., 1990), while the negative element is located in the gene body between position +4 and +34 (Chen et al., 1990). The positive element has been shown to be required for optimal Pit-1 promoter activity (McCormick et al., 1990). In addition, DNase footprinting analysis has revealed that the cAMP-regulated transcription factor CREB binds to two sites in the Pit-1 promoter between nucleotide -200 and -150 (Chen et al., 1990; McCormick et al., 1990): One CREB binding element (CRE) is palindromic and located between nucleotide -200 to -193, while the other is non-palindromic and located between -157 and -150 (see **Fig. 5.1A**) (McCormick et al., 1990). Forskolin induction has been mapped to this region (McCormick et al., 1990). Recently, Sanchez-Pacheco et al. reported that the region of the rat Pit-1 promoter

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containing the two CRE's is also responsible for repression of Pit-1 gene expression by thyroid hormone (Sanchez-Pacheco et al., 1995). In another study, Jong et al. have shown that either a phorbol ester or dexamethasone alone induces a 2-3 fold stimulation of Pit-1 promoter activity, while together these agents exhibit synergism. This synergistic effect also requires the region of the Pit-1 promoter containing the two CREs (Jong et al., 1995).

Although FSK and a phorbol ester have been shown to regulate Pit-1 promoter activity, no one has yet investigated the possibility that either PACAP or TRH can stimulate activity of this promoter. In this chapter, I have investigated this question.

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MATERIALS AND METHODS

Constructs

Plasmids (-200)Pit-1-CAT and (-200)Pit-1(ΔPitB1)-CAT were kindly provided by Dr. Michael Karin (McCormick et al., 1990). In the original description of these constructs, they were called Δ5'-200GHF1-CAT and Δ5'-200.1GHF1-CAT, respectively. (-200)Pit-1-CAT contains the first 200 bp of the proximal Pit-1 gene promoter region cloned in front of the CAT gene. (-200)Pit-1(ΔPitB1)-CAT is same as (-200)Pit-1-CAT, except that it contains a mutation in the Pit-1 binding site that prevents Pit-1 binding (McCormick et al., 1990).

Methods

All as described in previous chapters.

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RESULTS

As illustrated in **Fig. 5.1A**, the first 200 bp of the Pit-1 promoter contains two CRE's (CRE1 and CRE2) and a single Pit-1 binding site (PitB1) (McCormick et al., 1990; Chen et al., 1990). Since only one Pit-1 binding site exists upstream of the transcription start site of the Pit-1 proximal promoter, the Pit-1 promoter provides a useful model to study the function of the Pit-1 binding site in mediating hormonal regulation. I first examined whether TRH or PACAP could regulate (-200)Pit-1-CAT activity. As shown in **Fig 5.1,** TRH stimulated Pit-1 promoter activity 8-fold, while PACAP stimulated Pit-1 promoter activity 18-fold. In agreement with previous results (McCormick et al., 1990), FSK also yielded a strong stimulation of Pit-1 activity. Thus, either TRH or PACAP strongly stimulate cellular activity of the Pit-1 promoter.

As described above, previous studies have shown that the Pit-1 binding sites within the Pit-1 gene are important for its transcription (Chen et al., 1990; McCormick et al., 1990). Mutation of the Pit-1 binding site located between -70 and -38 abolishes its positive autoregulatory effect (McCormick et al., 1990; Chen et al., 1990). Previous studies have implicated Pit-1 binding sites in regulation of PRL promoter activity by either TRH (Yan et al., 1991), FSK (**Fig. 4.1**), or PACAP (data not shown). I therefore decided to investigate whether the Pit-1 binding site in the Pit-1 promoter is required for stimulation by TRH, FSK and/or PACAP. TRH stimulation of (-200)Pit-1-CAT was compared with stimulation of (-200)Pit-1(ΔPitB1)-CAT, in which the only Pit-1 binding site is mutated. It is seen in **Fig. 5.2** that TRH stimulated (-200)Pit-1-CAT activity about 10-fold, and that this induction was almost completely abolished by the mutation in the Pit-1 binding site. Furthermore, PACAP and FSK action were also inhibited by this mutation (**Fig. 5.3**). This later observation is in agreement with the

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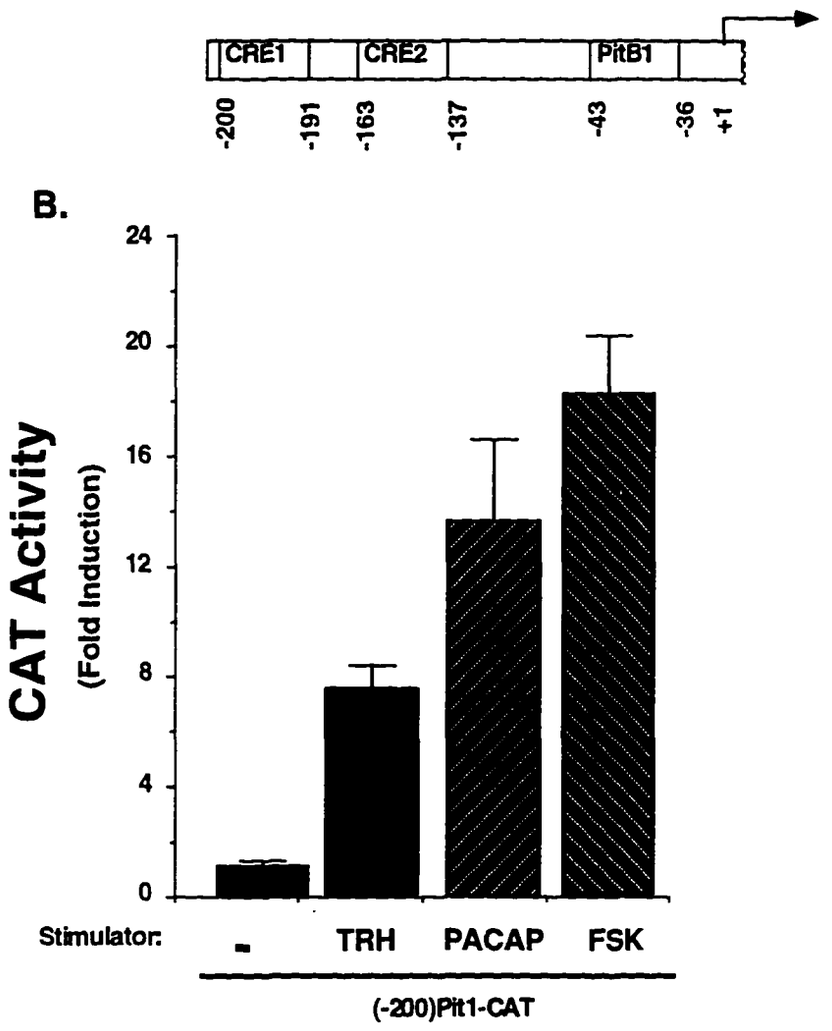

Either TRH or PACAP can stimulate Pit-1 promoter activity.
GH3 cells were transfected with 10 μg (-200)Pit1-CAT per cuvette ($6X10^6$ cells) by electroporation. The transfected cells were divided, and plated as described in Fig. 2.2. Following deinduction, the cells were incubated 18 hours ±FSK (1 μM), ±PACAP (100 nM) or ±TRH (100 nM) and assayed for CAT activity. Shown is stimulation (fold ± SD) by TRH, PACAP and FSK observed with duplicate dishes.

Figure 5.1

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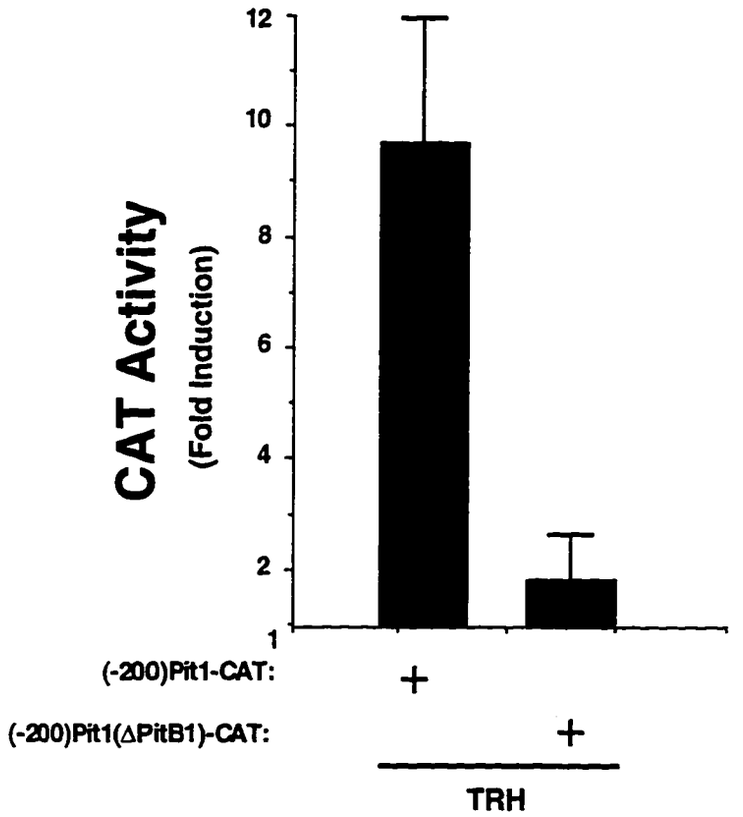

Stimulation by TRH of Pit-1 promoter activity is dependent upon an intact Pit-1 binding site. GH3 cells were transfected with 10 µg (-200)Pit1-CAT or (-200)Pit1(ΔPitB1)-CAT plus 10 µg tk-GH per cuvettee ($6X10^6$ cells) by electroporation. The transfected cells were divided, and plated as described in Fig. 2.2. Following deinduction, the cells were incubated for 18 hours ±TRH (100 nM) and assayed for CAT activity. Shown is TRH stimulation (fold ± SD) observed with triplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods in Chapter II.

Figure 5.2

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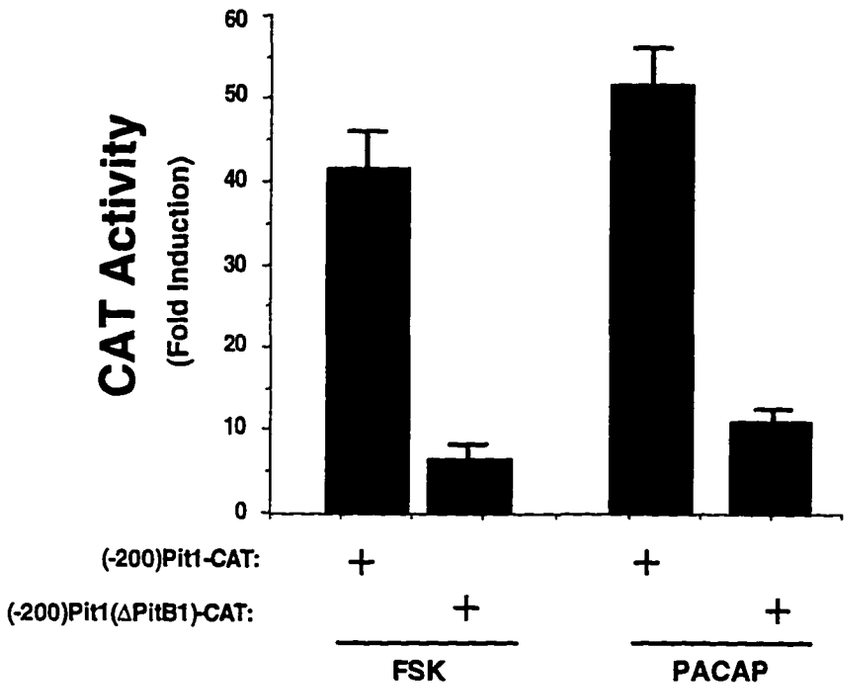

Stimulation by PACAP or FSK of Pit-1 promoter activity are dependent upon an intact Pit-1 binding site. GH3 cells were transfected with 10 μg (-200)Pit1-CATor (-200)Pit1(ΔPitB1)-CAT plus 10 μg tk-GH per cuvettee ($6X10^6$ cells) by the method of electroporation. The transfected cells were divided, and plated as described in Fig. 2.2. Following deinduction, the cells were incubated for 18 hours ±FSK (1 μM) or PACAP (100 nM) and assayed for CAT activity. Shown is stimulation (fold ± SD) by FSK or PACAP observed with triplicate dishes. Transfection efficiencies were normalized as described in Materials and Methods in Chapter II.

Figure 5.3

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finding that this mutation in the Pit-1 promoter inhibits stimulation by constitutively active α_s (Gaiddon et al., 1996).

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DISCUSSION

As shown in **Fig. 5.1**, PACAP can stimulate Pit-1 promoter activity. This observation provides a basis for the finding that FSK and PACAP increase Pit-1 mRNA levels in primary monolayer cultures of rat anterior pituitary cells (Soto et al., 1995). There are several lines of evidence suggesting that PACAP transcriptional effects in pituitary cells are mediated via the PKA pathway. First, PACAP can activate adenylate cyclase and increase cAMP levels in GH3 cells (Coleman and Bancroft, 1993). There are two cAMP response elements in the Pit-1 proximal promoter, and cAMP can stimulate the activation of this promoter (McCormick et al., 1990; Chen et al., 1990). Second, PACAP does not increase stimulation of the Pit-1 promoter over that yield by FSK (data not shown). Third, both FSK and PACAP induction of the Pit-1 promoter are dependent upon the Pit-1 promoter Pit-1 binding site (**Fig. 5.3**). Stimulation of the Pit-1 promoter by constitutively active Gαs and FSK, which have been shown both to be mediated by PKA, is also dependent upon this binding site (Gaiddon et al., 1996).

As shown in **Fig. 5.1**, TRH can also stimulate Pit-1 promoter activity. I have carried out a series of preliminary experiments to characterize this effect further (data not shown). The finding that TRH and PACAP have synergistic effects on (-200)Pit-1-CAT expression suggests that their actions are mediated via different pathways. My preliminary observation that the L-type Ca^{2+} channel blocker nifedipine blocks TRH stimulation of (-200)Pit-1-CAT activity suggests that this action of TRH is dependent on extracellular Ca^{2+}. I also observed that KCREB does not block TRH induction of (–200)Pit-1-CAT activity, suggesting that the PKA pathway is not involved in TRH stimulation of the Pit-1 promoter. My preliminary observation that the PKC inhibitor H-7 blocks the stimulation of the Pit-1 promoter by TRH raises the possibility that this

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action may be mediated via PKC, which is also Ca^{2+} dependent. This would differ from the pathway mediated by TRH to regulate the PRL promoter.

In summary, I have shown that TRH or PACAP can activate the Pit-1 promoter. To my knowledge, this is the first report showing that TRH can stimulate Pit-1 promoter activity. The Pit-1 binding site in the Pit-1 promoter is required for both TRH and PACAP induction. Future studies should be able to elucidate more completely the signal transduction pathways employed by TRH and PACAP to regulate the Pit-1 promoter.

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