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The acute regulation of POMC gene expression by the polypeptide hormone CRH in AtT20 cells

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The Acute Regulation of POMC Gene Expression by the Polypetide Hormone CRH in AtT20 Cells.

by

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Abstract

The Acute Regulation of POMC Gene Expression by the Polypeptide Hormone CRH in AtT20 Cells.

by

Dominique Lorang

Advisor: Professor James L. Roberts, Ph.D.

The goal of this thesis project is to elucidate the second messenger pathways mediating corticotropin releasing hormone (CRH)-regulated proopiomelanocortin (POMC) gene expression in the mouse AtT-20 D16/16 anterior pitiutary cell line. In this study we demonstrate that the polypeptide hormone CRH and the synthetic glucocorticoid dexamethasone differentially regulate POMC gene transcription in AtT20 cells similar to the rat corticotrope in primary culture. In order to examine the role of second messengers in the regulation of POMC gene expression by CRH, we measured the levels of POMC heteronuclear RNA (hnRNA) in nuclear RNA samples by a solution hybridization/nuclease protection assay and the rate of POMC gene transcription by a nuclear transcription run-on assay after short-term treatment with various Ca^{2+}- and cAMP-elevating secretagogues in AtT20 cells. In all run-on assays, the rate of POMC gene transcription reflected changes observed in POMC hnRNA levels measured under identical treatment conditions. Acutely elevating intracellular Ca^{2+} with the ionophore ionomycin or the dihydropyridine agonist Bay K is sufficient for activating POMC gene transcription and elevating POMC hnRNA levels with a magnitude of induction comparable to that elicited by cAMP-elevating agents. Therefore, both Ca^{2+} and cAMP are important second messengers regulating POMC gene expression. Combined Bay K and CRH treatment resulted in a greater increase in POMC hnRNA levels over treatment with either agent alone suggesting an interaction between

Ca^{2+}- and cAMP-related second messenger pathways. Activating the protein kinase C pathway with phorbol esters had no effect on endogenous POMC gene transcription or the expression of transiently transfected POMC-CAT fusion genes in AtT20 cells. In a series of POMC promoter/deletion experiments, we identified a Ca^{2+}/cAMP regulatory region of the POMC 5' flanking sequence located between -236 and -133 bp relative to the transcription initiation start site. Cytosolic elevations of either cAMP or Ca^{2+} stimulate POMC gene transcription to a lesser extent than does the combination of cAMP and Ca^{2+} through this discrete POMC promoter region, indicating an interaction between these signaling pathways perhaps at the level of binding of trans-acting factors to DNA.

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Introduction

The proopiomelanocortin (POMC) gene encodes the precursor protein to a variety of important neuromodulator pituitary peptides. The POMC gene is expressed in a variety of tissues in the mammal, with its main site of expression in the corticotrope cells of the anterior pituitary and the melanotrope cells of the intermediate lobe (Krieger et al., 1980). POMC gene transcription in the pituitary results in a single 1200bp mRNA product which is translated into an inactive precursor polypeptide by the usual process of ribosomal protein synthesis on the rough endoplasmic recticulum (see review, Lundblad et al., 1988) and packaged into granules in the Golgi Complex together with specific processing proteases. During the transport and storage of granules, the POMC precursor protein undergoes tissue-specific processing and posttranslational modifications to different biologically active secretory products in the two pituitary cell types: adrenocorticotropin releasing hormone (ACTH), b-endorphin and b-lipotropin are major products in the corticotrope, and the melanotropins (α-, β- and γ- MSH), CLIP and acetyl-β-endorphin in the melanotrope (Eipper and Mains, 1980) (Figure 1).

As part of the systemic response to stress, ACTH derived from the POMC polyprotein precursor, is released from the anterior pituitary into the general circulation. The release of ACTH is controlled by specific hypothalamic neurohormones (hypophysiotrophic hormones) known as releasing hormones, such as corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). They are synthesized in neurons of the paraventricular nucleus and exert their effects on the adenohypophysis following their secretion into the hypophyseal portal capillaries. Secreted ACTH ultimately acts on the adrenal cortex to regulate its metabolic activity. ACTH stimulates the synthesis and release of the glucocorticoids, corticosterone and cortisol (hydrocortisone), which ultimately feedback inhibit the hypothalamic-pituitary-adrenal axis, including CRH-induced synthesis and secretion of ACTH from corticotropes. The goal of this thesis is to investigate the molecular mechanisms involved in the regulation of POMC gene expression using a corticotroph model AtT-20 D16/16 cell line.

ACTH Secretion

It is well-established that the expression of the POMC gene in the corticotrope is positively regulated by CRH, a 41 amino acid peptide, isolated by Vale and colleagues (1981). It is the primary positive regulator of ACTH secretion from the anterior pituitary and AtT20 cell

(Hook et al., 1982). Neuropeptide activation of gene expression requires a model of signal transduction involving intracellular regulators "second messengers" which diffuse into the cell to activate a whole variety of cellular responses. The hypothalamic polypeptide hormone CRH specifically interacts with its membrane-bound cytoplasmic receptor, influencing several potential second messenger systems, including cAMP and Ca^{2+}.

CRH and the cAMP second messenger. The CRH-stimulated release of POMC-derived peptides in anterior pituitary and AtT20 cells is accompanied by an increase in adenylate cyclase activity and intracellular cAMP levels (Aguilera et al., 1983; Giguere etal., 1982; Labrie et al., 1982), suggesting the action of CRH is mediated primarily via the cAMP second messenger pathway. Receptor activation is coupled to the stimulation of an excitatory guanine nucleotide binding protein (Gs) which directly activates the inner plasma membrane-bound enzyme adenylate cyclase (Aguilera et al., 1983). This enzyme raises the level of cAMP in normal and transformed corticotrophs (Aguilera et al., 1986; 1983; Zatz & Reisine, 1985; Litvin et al., 1984; Miyazaki et al., 1984; Reisine, 84; Labrie et al., 1982), which determines the activity of a set of kinases including cAMP-dependent protein kinases (PKA).

CRH and cytosolic Ca^{2+}. Studies examining the regulation of Ca^{2+} movement and concentration in a cell have demonstrated that free cytosolic Ca^{2+} is one of several second messengers mediating receptor ligand-binding effects at the level of the plasma membrane to changes in neuropeptide gene expression in the nucleus. Numerous studies suggest the CRH receptor mediates its effects on POMC gene expression, in part, by enhancing cytosolic Ca^{2+} levels (Guild & Reisine, 1987; Abou-Samra et al., 1987; Imai & Gershengorn, 1986; Luini et al., 1985) in addition to elevating cAMP. In AtT20 cells, CRH causes a rapid rise in cytosolic Ca^{2+} concentrations, as measured with the Ca^{2+}-fluorescent dye Quin 2 (Reisine & Guild, 1987) and the whole cell patch-clamp technique (Hamill, O.P et al., 1981), in addition to inducing cAMP synthesis (Luini et al., 1985). Forskolin, the direct activator of adenylate cyclase and potassium, also increases cytosolic calcium levels in AtT20 cells (Guild & Reisine, 1987), and the cAMP analog 8BrcAMP produces an increase in Ca^{2+} conductance in patches of AtT20 cells (Luini et al., 1985). Once CRH increases cytosolic Ca^{2+} levels in AtT20 cells, Ca^{2+} remains elevated for as long as the cells are exposed to the stimulant (Reisine & Guild, 1987).

Ca^{2+} and ACTH secretion. An increase in intracellular Ca^{2+} in pituitary cells causes an immediate release of neuropeptides by a variety of methods used to evoke a rise in Ca^{2+}, such as depolarization, calcium ionophores, calcium channel agonists or hormones (Stojilkovic et al., 1988; Won & Orth, 1990a,b; Dave et al., 1987; Heisler, 1985; Tan and Tashjian, 1984). In [45]Ca^{2+} flux studies, neuropeptide release can be activated by micromolar Ca^{2+} fluxes in cells induced by membrane depolarization. This cytosolic Ca^{2+} accumulation is very fast, preceding hormone release earlier than the secretory response (see review, ref). Ca^{2+} entry is inhibited by inorganic (La^{2+}, Co^{2+} and Mg^{2+}) and organic (nifedipine) calcium channel blockers and is enhanced by the channel agonist Bay K 8644, indicating that Ca^{2+} enters the cell cytosol mainly through VGCC. Numerous studies have demonstrated that blocking the flux of Ca into cells with VGCC antagonists, inhibits CRH-stimulated ACTH release in pituitary monolayer cultures (Sobel, 1986; Abou-Samra et al., 1987).

CRH and ACTH secretion. Insertion of the protein kinase A (PKA) inhibitor into permeabilized AtT20 cells via a liposome technique greatly attenuates CRH and forskolin-stimulated Ca^{2+} mobilization, although it does not alter the rise in cytosolic Ca^{2+} induced by potassium (Guild & Reisine, 1987). Therefore, CRH elicits an increase in cytosolic Ca^{2+} concentrations through a cAMP-dependent process requiring the activation of PKA. On the other hand, potassium stimulates ACTH release by transiently opening voltage-gated calcium channels (VGCC), increasing Ca^{2+} entry without inducing cellular cAMP levels (Reisine, 1984) or activating PKA (Litvin et al., 1984) in AtT20 cells. The calcium ionophore A23187 also stimulates ACTH release without PKA activation, further supporting the view that activation of PKA (and, by inference, a change in cAMP-dependent protein phosphorylation) is not the final biochemical event that triggers hormone secretion (Miyazaki et al., 1984). A number of reports have also shown that cAMP and Ca^{2+} can act synergistically upon hormone secretion (Guild et al., 1986), as has recently been supported by the ability of elevated cytosolic cAMP to enhance Ca^{2+} entry through VGCC (see review by Miller, 1987).

VGCC are targets of PKA activity. Electrophysiological measurements of Ca^{2+} currents in pituitary cells have provided evidence for cAMP-dependent regulation of VGCC, as originally decribed in cardiac cells (Curtis and Catterall, 1985). PKA phosphorylates dihydropyridine (DHP)-sensitive calcium channel to increase its activity. This phosphorylation-dependent ion channel activation is absolute, since purified preparations of the catalytic subunit of PKA and ATP (or ATPgS) act directly on individual

Ca^{2+} channels in single-channel records from inside-out membrane patches of anterior pituitary derived GH3 tumor cells (Armstrong and Kalman, 1987; Armstrong and Eckert, 1987) and AtT20 cells (Phillips & Tashijian, 1982). The cAMP analog 8-bromo-cAMP also increases VGCC activity in voltage-clamped patches of intact AtT20 cells (Luini et al., 1985), further indicating that VGCCs are under the stimulatory influence of the PKA. A number of studies have proposed that through this mechanism, adenylate cyclase and VDCC may possibly regulate POMC gene expression in a concerted fashion (see review, Lundblad & Roberts, 1988).

CRH and POMC gene expression. CRH has direct stimulatory effects on the POMC gene in the anterior pituitary. The work from Roberts laboratory (Roberts et al., 1987; Lundblad et al, manuscript in preparation) as well as others have demonstrated that POMC mRNA levels are elevated by CRH in vivo (Loeffler et al., 1985; Dave et al., 1987) and in vitro (Affolter & Reisine, 1985; Reisine et al., 1985). POMC mRNA is maximally elevated 2- to 3-fold in AtT20 cells treated with CRH for 8hrs (Affolter & Reisine, 1985). Long-term administration of exogenous CRH *in vivo* for greater than 3 days results in an increase in POMC mRNA levels (Autelitano et al., 1988; Bruhn et al., 1984; Hollt et al, 1982; Lundblad & Roberts, 1988) and is paralleled by an increased secretion of POMC-derived peptides and total cell content of ACTH relative to controls (Notake et al., 1983). Long term treatment of cultured anterior lobe with pharmacological agents that increase cytosolic cAMP levels without activating the CRH membrane receptor, such as forskolin or 8-bromo-cAMP, also stimulates POMC mRNA levels 2-fold over controls (Loeffler et al., 1986).

In studies examining the long-term effects of secretagogues on POMC gene expression, it is uncertain whether a rise in POMC mRNA level results from increased mRNA stabilization or from increased POMC gene transcription or both. Transcriptional effects can be accurately measured in a nuclear transcription run-on (run-off) assay, in which nascent RNA transcripts are quantitated. It has been demonstrated that physiological concentrations of CRH rapidly (60min) induces changes in POMC gene transcription in the anterior lobe (Roberts et al.,1987; Gagner & Drouin, 1987). In fact, physiological concentrations of CRH (1nM) produce a 4- to 13-fold induction of POMC gene transcription well before any increases in POMC mRNA can be detected. This direct stimulatory effect of CRH on transcription is analogous to GRH induction of growth hormone transcription in cultured anterior lobe cells (Barinaga et al., 1985).

Increasing POMC gene transcription rapidly elevates POMC primary transcript in the nucleus, leading to increased levels of mature mRNA in the cytoplasm after several hours. Roberts and coworkers, employing solution hybridization/nuclease protection assays in conjuction with POMC intron/exon junctional probes, have demonstrated the rapid effects of CRH on POMC mRNA precursor levels in nuclei. Following injection of exogenous CRF into rats, the levels of anterior pituitary POMC primary transcript were induced (Autelitano, et al., 1988). Levin et al. (1989) demonstrated a significant increase in POMC primary transcript after 30 min incubation of anterior pituitary cultures with 0.5nM CRH. These increases in the accumulation of primary transcript accurately reflect alterations in gene transcription, as demonstrated by nuclear transcription run-on assays in rat anterior pituitary cultures (Eberwine et al., 1987; Gagner & Drouin, 1987).

Biochemical and immunological techniques have characterized PKA in AtT20 cells and cultured anterior pituitaries, and PKA has been shown to phosphorylate several intracellular macromolecules (Miyazaki et al., 1984; Litvin et al., 1984; Erlichman et al., 1983). The requirement for PKA in mediating the effects of CRH has recently been demonstrated by delivering the PKA inhibitor (PKI) into the cytosol of AtT20 cells via a liposome entrapment method (Reisine et al., 1985). Cells receiving PKI do not respond to CRH by increased ACTH release. PKI also blocks the induction of POMC mRNA by CRH and 8brcAMP, indicating an important role for PKA in the regulation of POMC gene expression. Enhanced PKA activity is also correlated with the phosphorylation of four distinct nuclear proteins within 5 min of CRH stimulation and dephosphorylation within 45 min (Reisine et al., 1985). The mechanisms by which cAMP and PKA activation exert their stimulatory effect on the POMC gene transcriptional apparatus, however, remain to be elucidated.

Calcium Physiology

Mobilization of intracellular Ca^{2+}. There are a number of mechanisms by which various types of extracellular stimuli can be transduced as a rise in cytosolic Ca^{2+}. Recepotor regulation of cytosolic Ca^{2+} generally involves two types of receptors: phospholipase C-linked receptors, such as arginine vasopressin, catecholamine and TRH receptors or PKA-linked receptors, such as the CRH receptor. The activation of PLC-linked receptors elicits a rapid elevation of cytosolic Ca^{2+} mobilized from intracellular and extracellular pools. Elevated intracellular Ca^{2+} levels are derived, in part, from a receptor-operated transduction process, involving a PLC-catalyzed hydrolysis of membrane

phosphoinositides. A GTP-binding protein (Gp)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) yields two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 is a ubiquitous intracellular mediator that acts, in part, by liberating Ca^{2+} stored within an intracellular nonmitochondrial pool (Berridge and Irvine, 1984). An accompanied rise in cytosolic Ca^{2+} and activation of diacylglycerol synergistically activates protein kinase C (PKC), another Ca^{2+}-activated phospholipid-dependent kinase .

There are also voltage-independent cation channels, which are not selective for Ca^{2+} and permeate both monovalent and divalent cations. These channels are regulated by second messengers generated in phosphoinositide turnover (Irvine & Moore, 1986; Morris et al., 1987; Kuno & Gardner, 1986). For example, second messenger-operated channels have been described in T lymphocytes. Kuno et al (1986) have demonstrated that mitogenic lectins increase the opening probability of cation channels in these cells. Mitogenic lectins stimulate the hydrolysis of PIP2 and the resulting increased formation of IP3 has been shown to activate the cation channel. It is not yet clear whether this mechanism of increased calcium entry through voltage-independent channels is common to other cells.

Receptors coupled to Ca^{2+} turnover. Receptors may regulate Ca^{2+} gating either positively or negatively without altering phosphoinositide turnover by at least four mechanisms of calcium gating (see review, Kojima, 1990). 1) A Ca^{2+}-permeable channel may be located in the receptor molecule and binding of the receptor leads to activation of the channel and the influx of Ca^{2+}. For example, in hippocampal cells, N-methyl-D-aspartate (NMDA), a specific agonist of a glutamate receptor subtype, stimulates Ca^{2+} entry by activating a Ca^{2+}-permeable channel which is considered to be associated with the NMDA receptor (Jahr & Stevens, 1987). 2) GTP-binding proteins may act as a transducer in regulating the channel by a receptor. For example, in the heart, b-adrenergic stimulants induce the Ca^{2+} influx through a Gs-dependent activation of VGCC (Yatani et al., 1987), a mechanism referred to as G protein-gated ion channels. 3) An intracellular messenger not associated with phosphoinositide turnover, perhaps generated by a G protein, may modulate the opening of the channel. In GH3 tumor cells, for example, vasointestinal peptide (VIP) mobilizes cytosolic Ca^{2+} by a cAMP-dependent phosphorylation of VGCC (Luini et al., 1985). 4) Changes in membrane potential may indirectly regulate voltage-gated calcium channels (VGCC). In adrenal chromaffin cells, for example, acetylcholine indirectly stimulates Ca^{2+} influx by opening VGCC (Noda et al., 1982). The subunits of the nicotinic receptor form a sodium channel, and receptor binding results in opening of the

channel gate and entry of sodium ions into the cell. Sodium influx depolarizes the plasma membrane, which in turn activates VGCC.

Kinetics of Ca^{2+} release. The properties of Ca^{2+} release are spatially and temporally complex (Berridge & Irvine, 1989; Parker & Ivorra, 1990). Ca^{2+} may be mobilized over a time-frame ranging from seconds to minutes as a result of second messenger-mediated events or more rapidly from milliseconds to seconds through the opening of ion channels. In a recent study, the measurment of Ca^{2+} release from highly localized (micrometer) regions of a single intact cell (oocyte) were performed by the combined use of flash photolysis of caged IP3 with confocal fluorescence Ca^{2+} monitoring (Parker & Ivorra, 1990). Photo-released lysis of IP3 in oocytes preloaded with caged IP3 resulted in a localized rise in cytoplasmic Ca^{2+}. Parker & Ivorra (1990) showed directly that localized Ca^{2+} liberation within a single cell is quantized (all or none) with increasing amounts of IP3 and indicated further that oscillations in Ca^{2+} are generated independently and asynchonously at different locations within the cell. Although the IP3-sensitive Ca^{2+} pool may be a collection of independent localized comparments that release Ca^{2+} in an all-or-none manner, the identity of the quantal Ca^{2+} release unit and the mechanism underlying the local quantal release still remain unclear.

Ca^{2+}-binding proteins. Once cytoslic Ca^{2+} raises in a cell, a number of proposed cell targets are activated. A growing number of abundant proteins interact with Ca^{2+} and sometimes with other second messenger systems. At least four Ca^{2+}-binding proteins are abundant in various types of nerve cells: calbindin-D23K, calretinin, parvalbulin and calmodulin. The function and the physiological roles for many of these calcium-binding protein families need to be investigated, including their function in different sets of neurons. So far the *in vitro* Ca^{2+} affinity and *in vivo* abundance of calbindin suggest that it should bind 99% of the free Ca^{2+} entering the cytosol and must therefore be an effective Ca^{2+} buffer. Calbindin-D23K is postulated to act as a Ca^{2+} ferry, facilitating the transport of Ca^{2+} through cells and maintaing cytosolic free Ca^{2+} below toxic level.

Calmodulin and Calmodulin-dependent kinase. Calmodulin, the best characterized Ca^{2+}-binding protein to date, has been implicated in a variety of regulatory funtions (Cheung, 1980). It has been reported to regulate the secretion of hormones, including ACTH by rat anterior pituitary cells (Sobel, 1986; Murakami et al., 1985; Aguilera et al., 1986). Calmodulin exerts pleiotropic effects on various cellular function by activating multiple enzymes and regulating various subcellular structures such as

ion channels. The molecular dissection of the early steps in the cellular response to increases in cytosolic Ca^{2+} involving calmodulin include the following: 1) Ca^{2+} binding to calmodulin, 2) calcium dependent conformational changes and intramolecular signalling within the calmodulin molecule; and 3) activation of enzymes that have calmodulin as a Ca^{2+} response element, such as the Ca^{2+}-dependent phosphodiesterase, the myosin light chain kinase, the plasma membrane Ca^{2+}-ATPase (Schatzmann, 1982) and calmodulin-dependent protein kinase II (see review, Rassmussen & Means, 1985). Because calmodulin is the Ca^{2+} responsive element of multiple protein structures within the same cell, it provides a common motif to one set of pathways to a cell's response to transient rises in intracellular Ca^{2+}.

Voltage-gated calcium channels. Cells display spontaneous action potential activity as a result of the activation of plasma membrane VGCC and/or sodium channels. These channels are closed when a cell is at rest but open during action potentials; thus modulators of the activity of these channels affect the amount of Ca^{2+} entering the cell over time. The measurement of Ca^{2+} currents recorded from single nerve cells has been shown to be kinetically and pharmacologically complex, consistent with the presence of multiple VGCC (for review, see Fox et al., 1988). Single channel studies in neural and endocrine cells demonstrate that step depolarizations of the membrane from negative holding potentials to near 0mV evoke inward currents that fall into three conductance classes. These current types can be further characterized by their inactivation characteristics and pharmacological profiles as exhibited in Table 1 (Tsien et al., 1987).

Classes of VGCC. The L channel (Nowycky et al., 1985a) is a 25-pS channel, which exhibits a high threshold for activation (approximately 20mV), mediates long-lasting Ca^{2+} currents and is a target for dyhydropyridine (DHP) pharmacological agents. The probability for channel opening decreases in the presence of DHP antagonists such as nifedipine, while DHP agonists such as Bay K 8644 promote L-type channel opening (Fox et al., 1987a, b). The two other calcium channel types, termed T and N, have smaller single-channel conductances (8 and 13pS, respectively) and are largely inactivated at the resting potential. The T channel can be further distinguished in that it exhibits a threshold approximately 40mV lower (-60mV) than the other channel types. Unlike the L channel, the function of the T and N channels is unaffected by DHPs. Other pharmacological agents are known to inhibit these channels, such as cadmium and the snail toxin, w-Conotoxin GVIA.

VGCC in corticotropes. The VGCC of AtT20 cells have been carefully characterized as to their electrophysiological properties, sensitivity to drugs and role in hormone secretion. AtT20 cells have at least two types of VGCC, L and T channels (Suprenant, 1982). The calcium channels present in AtT20s appear to resemble those in other excitable cells with respect to their sensitivity to conventional Ca^{2+} antagonists. The two types of Ca^{2+} channels, fast deactivating or high threshold (L-type) and slow deactivating, low threshold or transient (T-type), were distinguished on the basis of their different rates of deactivation, the voltage range over which they activate, their activation time course, inactivation proerties, Ca^{2+}/Ba^{2+} conductivity and stability under whole-cell recording. In AtT20 cells, membrane depolarization with K^+ or with sodium channel activators causes an ingress of Ca^{2+} through plasma membrane calcium channels (Guild & Reisine, 1987; Sabol, 1980; Simantor, 1978) and an increase in ACTH release which is blocked by VGCC or sodium channel antagonists (von Dreden et al., 1988; Sabol, 1980; Richardson & Schonbrunn, 1981; Adler et al., 1981).

Ca^{2+} Regulated Gene Expression

The regulation of neural gene expression by membrane depolarization and synaptic activity has demonstrated the importance of calcium as an intracellular mediator (Black et al., 1985; Greenberg et al., 1986; Kley et al., 1986; Morgan & Curran, 1986). In primary cultures of adrenal chromaffin cells, for example, proenkephalin mRNA levels have been shown to increase in response to K- or veratridine-induced depolarization (Kley et al.,1987; Waschek et al., 1987) and calcium ionophores (Kley, 1988) suggesting that one of the biological roles for VGCC on neuronal cell bodies may be in the transduction of depolarizing signals to the nucleus to regulate gene expression. In support of this concept, recent studies have demonstrated that membrane depolarization causes not only rapid alterations in protein phosphorylation (reviewed in Nairn et al., 1985), but also activation of new programs of gene expression in neurons (Bartel et al., 1989). The molecular mechanisms by which genes are regulated by membrane depolarization is poorly understood, though for some genes it has been shown to be dependent on the mobilization of calcium ions into cells (Greenberg et al., 1986; Morgan & Curran, 1986; Bartel et al., 1989).

Ca^{2+} and POMC gene expression. Although Ca^{2+} is important in POMC-derived peptide secretion, very little is known about its role in the regulation of POMC gene transcription. Numerous studies have examined the long-term effects of a variety of Ca^{2+} mobilizing agents at the level of POMC mRNA accumulation. Long-term treatment of

AtT20 cultures with the ionophoretic molecule A23187, which permeabilizes cellular membranes to Ca^{2+} independent of endogenous membrane channel activation (Rasmussen et al., 1984), induces POMC mRNA levels and POMC-derived peptide secretion (Reisine et al, 1985). The DHP activator Bay K 8644 also increases POMC mRNA levels, and augments the stimulatory effects of forskolin in primary pituitary cultures (Loeffler et al., 1986) and AtT20 cells (von Dreden et al., 1988). A23187 also consistently upregulates the rate of POMC gene transcription in primary cells (Eberwine et al., 1987) further suggesting the involvement of Ca^{2+} as a putative second messenger mediating rapid changes in POMC gene transcription. Membrane depolarization with K^+ or with sodium channel activators, which causes an ingress of Ca^{2+} through plasma membrane calcium channels (Guild & Reisine, 1987; Sabol, 1980; Simantor, 1978) also increases POMC mRNA levels which is blocked by VGCC or sodium channel antagonists (von Dreden et al., 1988).

The source of Ca^{2+} mediating this regulatory effect on POMC gene expression has been investigated by a number of laboratories. In one study, anterior pituitary cells were cultured in Ca^{2+}-free medium for 24 hrs in order to determine whether extracellular Ca^{2+} was necessary for POMC gene expression. Under these treament conditions, both POMC and prolactin mRNA levels were reduced 50% relative to controls (Dave et al., 1987), suggesting basal activity could be maintained by internal calcium stores alone.

Since long-term deprivation of extracellular Ca^{2+} may result in adverse nonspecific effects on gene transcription, another method for examining the effects of external Ca^{2+} on neuropeptide gene expression involves blocking the flux of Ca^{2+} into cells with VGCC blockers. However results obtained with VGCC blockers are also contraversial because of potential side effects unrelated to calcium channel activity. In one study, POMC mRNA levels were shown to be reduced approximately 50% relative to untreated controls in anterior pituitary cultures treated with the Ca^{2+} channel antagonists D600, verapamil or nifedipine for 48 hrs (Loeffler et al., 1986). In an AtT20 cell study, 48 hr treatment with verapamil (10uM) blocked the Bay K-stimulatory effects on POMC mRNA levels, eventhough in the same experiment it did not block the stimulatory effects of CRH (100nM) or 8-bromo-cAMP (1mM) (von Dredon et al., 1988). Another organic calcium channel antagonist, D600, was shown to have no significant effect on basal POMC or prolactin mRNA levels in anterior pituitary cultures after 24 hrs, eventhough ACTH release was blocked (Dave et al., 1987). These varying effects of VGCC blockade on POMC gene expression may be accounted for by a number of factors, including duration of drug

treatment, amount of time cells are cultured and differences in cell response between normal and tumor corticotropes.

Ca^{2+}-dependent regulation of other genes. A number of studies have demonstrated that prolactin gene expression is positively regulated by Ca^{2+}. Calcium ionophores increase the rate of prolactin gene expression in GH3B6 cells (Laverriere et al., 1988) and increase the level of prolactin mRNA and peptide synthesis upon addition of extracellular Ca^{2+} to GH3 cells grown in Ca^{2+}-free medium (White et al., 1981; Bancroft et al., 1985). Ca^{2+} has also been shown to act synergistically with TRH and EGF to increase prolactin mRNA levels (White & Bancroft, 1983), and calcium blockers rapidly reduce the increase in transcription obtained with these peptide hormones (Murdoch et al., 1985). Treating GH cells with organic calcium channel blockers, also inhibits prolactin synthesis and mRNA levels without affecting overall protein synthesis (Hinkle et al., 1988). Conversely, the calcium channel agonist Bay K 8644 or partial membrane depolarization with potassium augments prolactin mRNA levels and peptide synthesis.

On the other hand, Ca^{2+} does not appear to regulate the expression of the closely related pituitary growth hormone (GH) gene. Neither the DHP calcium channel agonist or antagonists alter GH synthesis (Enyeart et al., 1987, Hinkle et al., 1988), and calcium depletion does not affect endogenous GH mRNA levels or peptide synthesis (White et al., 1981; Bancroft et al., 1985). Therefore, within the anterior pituitary gland, calcium-regulated gene expression is cell-type specific.

Elevating cytosolic Ca^{2+} by membrane depolarization has also been shown to regulate neural gene expression (Greenberg et al., 1986; Kley et al., 1986; Morgan & Curran, 1986). One of the best-studied models of neural gene regulation by depolarization is the proenkephalin gene (Van Nguyen et al., 1990). In PC12 and C6-glioma cells stably transfected with a proenkephalin (-193 to +70) CAT fusion plasmid, K^+-evoked membrane depolarization induces CAT activity in a dose-dependent and time-related fashion. The level of activation of the fusion gene is proportional to extracellular Ca^{2+} concentration and is inhibited by verapamil, suggesting Ca^{2+} is the effector for the transcriptional response to depolarization. In addition, stimulation of the transfected proenkephalin gene by K^+ or the calcium ionophore A23187 is synergistic with cAMP-elevating agents (Kley et al., 1986; Waschek et al., 1987; Kley, 1988) eventhough treatment with KCl or A23187 alone has little or no effect. This synergistic effect cannot be a result of superinduction of cAMP since neither K^+-induced depolarization or A23187 has an effect on cAMP levels.

Interestingly, the effects of K^+ and A23187 were inhibited by the calmodulin antagonists W7 and trifluoperazine, while the stimulatory effect of the adenylate cyclase activator forskolin was not. These studies indicate that proenkephalin gene expression involves an interaction between cAMP and Ca^{2+}, supporting that cells can integrate multiple intracellular signals to regulate gene expression.

Ca^{2+}-responsive DNA elements. A number of investigators have examined a possible interaction between the cAMP and Ca^{2+} second messenger systems in transcriptional activation by mapping the 5' flanking sequences of genes regulated by both cAMP- and Ca^{2+}-elevating agents. So far, calcium-responsive elements (CaRE) have been reported in several genes. A calcium-inducible regulatory region has been isolated from the rat genome (Lin et al., 1986), with no obvious homology to cAMP consensus sequences. A calcium-responsive sequence -TGACGTTT- has been identified in the *c-fos* gene at -72 to -54 bp (with respect to the start of transcription). This element is similar to the consensus 8 bp palindromic cAMP regulatory element (CRE) TGACGTCA which has independently been shown to confer transcriptional regulation by cAMP (Sassane-Corsi et al., 1988; Fisch et al., 1989). Growth factor genes containing 5' flanking Ca/CRE-like elements include nur/77 (Watson & Milbrandt, 1989) and zif/268 (Changelain et al., 1989) both of which are induced to high levels by depolarization. Neuropeptide genes, such as somatostatin (Montminy at al., 1986; Montminy & Bilezikjian, 1987) and vasoactive intestinal peptide (Tsuada et al.,1987) and proenkephalin (Comb et al., 1986) have been shown to be regulated by depolarization and Ca^{2+}. Depolarization-induced activation of the proenkaphalin gene is conferred by a previously characterized cAMP-inducible enhancer. These results suggest that cAMP and Ca^{2+} responsiveness converge on a single DNA element, suggesting some CREs may also be CaREs.

Greenberg and colleagues (1990) have begun to dissect the biochemical pathway mediating the transcriptional induction of the proto-oncogene *c-fos* gene by membrane depolarization and Ca^{2+} influx. They have shown that the *c-fos* CaRE is functionally indistinguishable from a CRE, and is therefore called a Ca/CRE. In addition, regulation of Ca/CRE by calcium and cAMP cannot be dissociated by single bp mutagenesis, further suggesting that both second messengers use a common, rather than distinct, target protein(s) that interact with the CaRE. The final steps in the depolarization/calcium signalling pathway of *c-fos* gene transcription are, in fact, common to the pathway of gene activation by cAMP, involving phosphorylation of the transcription factor CREB and interaction of CREB with CaRE. Interestingly, stimulated *c-fos* transcription in PC12 cells by KCl depolarization or

treatment with the calcium ionophore A23187 has no effect on cAMP levels at 5 min or for as long as 60 min (Sheng et al., 1988; 1990; Griffith & Schulman, 1988), suggesting that *c-fos* induction by Ca^{2+} is not mediated by a secondary elevation of intracellular cAMP. Rather, Greenberg et al. (1990) suggest a model in which the transcription factor CREB bound to its cognate DNA sequence (Ca/CRE) functions as a bifunctional target for distinct cAMP- and Ca^{2+}-regulated protein kinases.

cAMP-Independent Regulators

Another class of ACTH secretagogues, arginine vasopressin (AVP), angiotensin II, oxytocin, the cholycystokinin C-terminal octapeptide (CCK-8) and NE, act in concert with CRH to modulate POMC gene expression. These neurohormones, by themselves, are weak stimulators of ACTH secretion in rat anterior pituitary cells in vivo (MacCann & Fruit, 1957; Yasuda et al, 1978) and in vitro (Fleisher & Vale, 1968). Their mechanisms of action involve the hydrolysis of phosphoinositides and the activation of protein kinase C (Abou-Samra et al., 86; Negro-Vila, 85) (Figure 3). In the presense of CRH, these neurohormones markedly potentiate CRH-stimulated secretion of POMC-derived peptides (Vale et al., 1983; Gilles & Lowry, 79; Gilles et al., 1982; Vale et al., 83; DeBold et al, 1984).

AVP and POMC-derived peptide secretion. There are several potential sites of modulation for the potentiation effects of AVP on CRF-stimulated cAMP accumulation: 1. PKC activators, such as phorbol esters, have been demonstrated to induce cAMP levels in certain cells, suggesting that phorbol esters upregulate transcription by enhancing adenylate cyclase or inhibiting phosphodiesterase activity. However, the AVP effects are not due to a direct action of the peptide on adenylate cyclase, since AVP does not potentiate adenylate cyclase activity in broken cells or pituitary homogenates (Abou-Samra et al., 1987; Gaillard et al., 1984). 2. PKC-linked hormones have been shown to enhance adenylate cyclase activity at a level distal to G proteins, such as by activating factors that directly stimulate Gs or the catalytic subunit (Bell et al., 1985). 3. Synergism may be due to a step subsequent to the generation of cAMP in those systems where phorbol esters fails to increase basal or forskolin-induced cAMP levels (Anderson et al, 1988). 4. cAMP degradation may be inhibited through cAMP-independent mechanisms via modulation of phosphodiesterase activity. 5. C kinase may couple receptors of certain cAMP-independent hormones to PI turnover or ion channels (Okajima & Ui, 1984; Hgashida et al., 1986; Lao et al.,1985; Abdul-Badi et al, 1987, ref 21, 22). 6. Arachidonic acid and its metabolites

have been found to participate in the mechanism of action of ACTH secretagogues (Vlaskovska et al., 1984; Vlaskoska & Knepel, 1984; Abou-Samra et al., 1986; Bitonti et al., 1980; Enna & Karbon, 1987) and may provide another point at which the intracellular action of CRF and AVP converge (Antoni, 1986). 7. Synergism may occur if cAMP enhances the effect of phorbol esters on C kinase activity, however this appears unlikely since there is no precedence in the literature.

In addition, species-dependent AVP effects exist further complicating the study of AVP-regulatory mechanisms in pituitary ells. In some species, AVP, and not CRH, is the most important POMC peptide releasing factor. For example, in isolated ovine anterior pituitary cells, AVP stimulates a greater release of ACTH than does CRF (Familari et al., 1988). In cultured mouse anterior pituitary cells, AVP does not modify CRH-stimulated accumulation of cAMP, which is where AVP is shown to potentiate CRH-induced accumulation of cAMP in rat pituitary cells (Abou-Samra et al., 1987). Instead, increases in phosphoinositide breakdown presumably through the activation of a hormone-sensitive phospholipase C are important (Castro et al., 1989).

AVP receptors in the pituitary. The cAMP-independent secretagogue AVP binds to unique specific receptor sites in the anterior pituitary of rats (Koch & Lutz-Bucher, 1985), similar to V1 or V2 types in the periphery (Baertschi & Friedli, 1985, Endo 116: 499-502; Knepel et al., 1984, Endo 114: 1797-1804). Direct evidence of coupling of pituitary AVP receptor with phospholipid turnover has been provided by experiments showing an increase in the incorporation of ^{32}P into phospholipids and the stimulation of phosphate product in pituitary cells incubated with VP (Raymond et al., 1985; Guillon et al., 1987; Todd & Lightman, 1987). The guanine nucleotide binding protein Gp couples the activation of the AVP receptor to PI turnover in cultured rat anterior pituitaries (Abou-Samra et al., 1987; Cockcroft & Gomperts, 1985; Baertschi, J. & Friedli, 1985; Todd & Lightman, 1987; Raymond et al., 1985; Raymond et al., 1985; Bilezikijian et al, 1987).

AVP and protein kinase C activity. Activating PKC with tumor-promoting phorbol esters or synthetic diacylglycerol mimicks the secretagogue effect of AVP in corticotrophs (Abou-Samra, 1986; 1987) and AtT20 cells. PKC translocates from the cytoplasm to a membrane-associated fraction in anterior lobe cells cultured with phorbol esters or DAG analogues (Phillips & Tashjian, 1982) and in AtT20 cells treated with phorbol dibutyrate (Zatz et al, 1987, Loeffler et al., 1989). Short-term incubation with AVP or PKC activators increase ACTH release in a dose-related manner without an effect on cAMP content in

cultured rat anterior pituitary (Abdu-Samra et al, 1987; Abou-Samra et al, 1986; Heisler, 1984; Philips & Tashjian, 1982; Phillips & Jaken, 1983; Murakami et al., 1984) and cultured mouse cells (Castro et al., 1989). Conversely, inhibition of PKC activation by retinal attenuates the potentiating effects of VP and phorbol esters on cAMP accumulation and inhibits release (Abou-Samra, 1987). Inhibition of endogenous PKC using prolonged incubation of cells with phorbol esters also results in the loss of immunoprecipitable PKC (Bilezikijian et al, 1987) abolishing the potentiating effects of AVP and PMA on CRH-stimulated cAMP production and secretion in primary cultures of rat anterior pituitary cells (Smith & Vale, 1981; Carvalla & Aguilera, 1989) and AtT-20 cells (Phillips & Jaken, 1983). Although PKC plays a major role in the mechanism of action of AVP on secretion, the mechanism of AVP potentiation of POMC gene transcription by CRH remains speculative.

AVP and POMC gene transcription. Although AVP induces POMC-derived peptide secretion and potentiates CRH-stimulated cAMPaccumulation, it would be predicted that AVP potentiates CRH-induced POMC gene expression. However a number of studies have shown that chronic treatment with AVP and CRH does not potentiate CRH-stimulated peptide content (Wand & Eipper, 1987) or cytoplasmic mRNA levels (Suda et al., 1989). Roberts and coworkers (Levin et al., 1989) have recently demonstrated that AVP alone does not regulate the transcription of the POMC gene in dispersed rat pituitary cells. AVP also does not potentiate short-term CRH-stimulated production of POMC primary transcript, suggesting AVP affects POMC gene expression after transcription (Levin et al., 1989). The effects of cAMP-independent activators on mRNA stability remains an open question, since the effects of AVP or phorbol esters on POMC mRNA levels in pituitary cells are contraversial in the literature (Levin & Roberts, 1991).

Glucocorticoids

Glucocorticoids, synthesized in the adrenal cortex, negatively regulate POMC gene expression in anterior lobe cells. The mechanism of action of glucocorticoids and other steroid hormones is different from that of neuropeptide hormones. Glucocorticoids do not have direct effects on cAMP levels, IP3 turnover and PKC activation. Their hydrophobic nature allows them to pass through the plasma membrane to interact with their specific protein receptors, which are located inside cells. The resulting steroid-receptor complexes undergo nuclear localization, and associate with genomic sites where they alter the

efficiency of transcriptional initiation at specific promoters (for review, see Yamamoto, 1985).

Glucocorticoids inhibit POMC gene expression. Circulating glucocorticoids act at both the hypothalamus to inhibit secretion of CRH and on the pituitary gland, via the portal capillary system, to suppress ACTH release. Since glucocorticoids may act indirectly via a product induced and secreted from another cell type in intact animals or dispersed pituitary cells, AtT20 cells have been a useful cell system for directly examining the in vitro effects of glucocorticoids in corticotrophs. In this tumor cell line, inhibition of secretion due to short-term glucocorticoid treatment (<4hrs) is not due to an increase in hormone degradation, a change in processing, a decrease in hormone synthesis or an interference with secretagogue binding (Phillips & Tashjian, 1982). Rather, glucocorticoids act, in part, by altering the mechanism of stimulated hormone release. In fact, brief exposure of AtT20 cells to dexamethasone blocks the stimulated release of ACTH without affecting either the intracellular content of ACTH or the capacity of cells to synthesize ACTH (Phillips & Tashjian, 1982). Reisine et al. (1984) have shown that short-term treatment of AtT20 cells with dexamethasone (0.1uM) attenuated the ability of both forskolin and isoproterenol to stimulate ACTH release, without an affect on the ability of A23187 to stimulate release. Interestingly, dexamethasone pretreatment did not alter the ability of either forskolin or isoproterenol to activate protein kinase A activity. Glucocorticoids alter the binding of fluorescent CRH (Schwartz et al., 1986), downregulate CRF receptor number, attenuate CRF-dependent ACTH secretion and cAMP accumulation.

In the anterior lobe of the pituitary, glucocorticoids rapidly inhibit transcription of the POMC gene, measured in a run-on transcription assay (Birnberg et al., 1983; Eberwine & Roberts, 1984; Gagner & Drouin, 1985), indicating that the glucocorticoid receptor may directly repress the POMC promoter as demonstrated in the a-subunit gene (Akerblom et al., 1988). Dex inhibits POMC-derived peptide synthesis resulting from a diminished level of POMC mRNA accumulation in the cytoplasm, with a longer time course of action than the inhibition of secretion (Roberts et al., 1979; Schacter et al., 82; and Herbert et al., 1981). Long-term treatment with dex decreases POMC mRNA levels in AtT20 cells (Reisine et al, 1982; Nakamura et al., 1978; Roberts et al., 1979) without an effect on basal or forskolin-stimulated PKA activity (Miyazaki et al., 1984). Transcriptional and secretory inhibition is rapid, occuring after 30min. POMC gene transcription rate in AL cultures cotreated with CRF and DEX was found to be intermediate between control levels and those of CRF alone (Gagner & Drouin, 85,87), suggesting the glucocorticoids

interfere with CRH-stimulated signal transduction, such as cAMP formation (Bilezikian & Vale, 1983; Giguere et al., 1982).

Glucocorticoids and proto-oncogenes. As transcription factors, glucocorticoids have been shown to act as positive regulators of gene expression in some situations and not anothers. In some genes, positive regulation has been attributed to binding to promoter elements known as glucocorticoid response element (GRE), and negative regulation to to binding to promoter elements different from the GRE. On the other hand, it has recently been shown that a single 25bp GRE in the upstream regulatory region of the mouse proliferin gene can either stimulate or repress transcription after binding of the glucocorticoid receptor with other transcription factors depending on the cellular context. Recent results indicate that specific receptor-DNA interactions are not the sole determinant of positive and negative regulation by glucocorticoids (Mordacq & Linzer, 1989; Sakai et al., 1988). Rather, negative regulation of neuropeptide gene expression by glucocorticoids appears to involve transcription factor (protein-protein) interactions between the glucocorticoid receptor and Fos/Jun, as well as the association of the steroid receptor with activated promoter elements. Yamamoto and colleagues (Diamond et al.,1990) have demonstrated that the mouse proliferin gene contains an upstream "composite" glucocorticoid response element (GRE) which confers positive or negative regulation by glucocorticoids. This 25bp GRE sequence binds selectively in vitro to the glucocorticoid receptor and to c-Jun and c-Fos, the components of the phorbol ester-activated AP-1 transcription factor, modulating the transcriptional efficiency of the proliferin gene. In fact, it appears the ratio of functional c-Jun and c-Fos expressed within a particular cell type determines the overall activity of a CRE/AP-1 containing promoter to glucocorticoids.

Glucocorticoids have also recently be shown to negatively regulate collagenase gene expression through direct protein-protein interations, as demonstrated by cross-linking and coimmuno-precipitation studies (Yang-Yen et al., 1990). Karin and colleagues (1990) demonstrate that this negative regulation is due to the direct repression of AP-1 activity (fos/jun) by the glucocorticoid receptor. The ligand binding domains of GR and the region including the leucine zipper of c-jun are required for this repression (Schule et al., 1990). In addition, the glucocortoid receptor and the transcription factor Jun/Ap1 can reciprocally repress one another's transcriptional activation independent of DNA binding (Schule et al.,1990).

Rat POMC Gene Promoter Structure

A large number of transcriptional control elements proximal to the transcriptional initiation start sites of numerous genes have been identified, including promoter elements responsible for the proper initiation of transcription (McKnight & Tijan, 1986) and enhancer elements that increase the transcription from a promoter and are responsible for tissue-specific gene expression (Maniatis et al., 1986; Dynan, 1989). Although a number of transcriptional elements exist within the 5' flanking region, the presence of these motifs is necessary but may not be sufficient to constitute a transcriptional responsive element (Deutsh et al, 1988). It is therefore hard to predict the signal-responsive properties of a gene by inspecting its sequence.

POMC gene structure. POMC genomic DNA has been isolated and sequenced from several mammalian species including rat (Drouin & Goodman, 1980), mouse (Uhler et al., 1983; Notake et al., 1983) and human (Takahashi et al., 1983). The structure of the rat POMC gene is schematically shown in Figure 1. The gene containes three exons separated by two large introns: approx. 3.5-4 kilobases (kb) for intron A and 2-3kb for intron B. The first exon is 100 bp in length and contains most of the 5' untranslated portion of the pituitary POMC mRNA. The second exon is 150bp and contains a small portion of the 5' untranslated sequence and the begining of the protein coding portion of the mRNA. The third exon encodes all the peptides such as ACTH, b-endorphin and the melanotropins.

The POMC 5' flanking region is quite complex. It is transcriptionally responsive to the stimulatory effects of peptide hormones and to the inhibitory effects of steroid hormones. The cAMP-, calcium- and PKC-dependent second messenger pathways have been proposed to modulate transcription in a concerted fashion. In addition, the gene is expressed in a tissue-specific fashion, indicating the presence of promoter elements that exhibits tissue-specific regulatory properties..

The 5' flanking region of the POMC gene confers tissue-specific expression in the pituitary (Tremblay et al., 1988; Jeannotte et al., 1987b). In transgenic mice bearing a POMCneo chimeric gene (-706 to +63bp POMC gene fused to the bacterial neomycin resistence coding gene), the tissue pattern of transgene expression almost completely parallels the endogenous expression of POMC, with high expression levels in the pituitary and hypothalamus (Tremblay et al., 1988). The specificity of the POMC promoter activity was also assessed in nonpituitary (L) cells and clonal AtT20 corticotrophs via gene transfer

studies. The rat POMC sequences necessary for tissue-specific promoter activity were narrowed to a smaller -480 and -34bp fragment (Jeannotte et al., 1987).

In other pituitary expressed genes, cell-specific activation have been shown to involve multiple cis-active elements. For example, the pituitary transcriptional activator Pit-1 is capable of directing tissue-specific expression of the rat PRL and GH gene (Bodner & Karin, 1987; 1988; Nelson et al., 1986; 1988). Interestingly, pit-1 has not been identified in the POMC gene despite the exclusive expression of Pit-1 mRNA in the anterior pituitary gland. Such tissue-specific transcriptional control mehanisms remain to be elucidated.

Hormone responsive elements. Roberts and colleagues (1987) analyzed the rat POMC gene promoter region for hormonally response elements through the use of fusion gene constructs in a transient expression system using AtT-20 cells. In cells transfected with a DNA construct containing the POMC promoter region (-704 to +63 nucleotides) fused to the structural gene for the bacterial chloramphenicol acetyltransferase (CAT) gene (POMC-CAT), synthesis of CAT enzyme is increased with CRH or forskolin incubation and decreased with glucocorticoid treatment (Figure 2).

AtT20 cells were also transiently transfected with POMC promoter fragments inserted 5' to the thymidine kinase (tk) promoter fused to CAT. Two functional POMC transcriptional regions, -478 to -320 and -236 to -133, were identified. Fragment -478 to -320 was shown to confer an elevated promoter activity and a 2-fold inducibility by CRH, and a smaller fragment, -320 to -133, confers a stronger inducibility (5 to 10-fold) by CRH. When the POMC 5' flanking sequence was analyzed for its ability to bind proteins present in the crude nuclear extracts of AtT-20 cells by *in vitro* footprinting by DMSO and DNase protection (Lundblad et al., in press), multiple footprints were identified throughout the promoter region. Interestingly, although the POMC gene is regulated by CRH and cAMP-related agents, none of the 5' flanking sequence has significant homology with the cAMP responsive element (CRE) of other cAMP-regulated genes.

Glucocorticoid-negative regulation. Negative regulation of transcription by glucocorticoids has been documented in many genes (Camper et al., 1985; Isreal & Cohen, 1985; Charron and Drouin, 1986; Fremeau et al., 1986; Frisch & Ruley, 1987; Weiner et al., 1987; Tashjian et al., J.Biol.Chem 47, 61-70). The GR-glucocorticoid complex exerts a direct effect on POMC gene transcription since the protein synthesis inhibitor cyclohexamide fails to block downregulated transcription by dex (Gagner & Drouin, 87).

Tissue-specific factors do not appear to be involved since POMC fusion genes introduced into murine mammary epithelial cells or murine fibroblasts are negatively regulated by dexamethasone (Nakamura et al., 1978).

Glucocorticoid responsive elements. DNase I, exonuclease-III footprinting and dimethyl sulfate protection analyses of the binding of purified rat liver GR to specific sequences in the POMC gene demonstrate greater than five GR binding sites in the promoter and downstream intervening sequences (Drouin et al., 1987). Deletion of the POMC gene fragment (-38 to -132bp) containing the -63bp GR binding site abolishes glucocorticoid repression and double point mutation at this site reduces GR binding and regulation in stable transfection (Drouin et al., 1989). However, these results are difficult to interpret since the site of integration of the POMC-neo DNA into the cell chromosome were not examined.

Roberts and colleagues have also studied glucocorticoid negative regulation of POMC promoter activity through specific DNA elements (Lundblad et al., in preparation). In a series of transient transfection studies using the steroid hormone receptor defient monkey kidney CV-1 cell line, POMC promoter deletions in the -38 to -132 5' flanking sequence were fused to the reporter CAT gene. A MMTV-CAT DNA construct was cotransfected as a control for transfection efficiency and glucocorticoid regulation. Interestingly, basal CAT activity was induced in deletion constructs relative to full promoter POMC-CAT activity, and glucocrticoid treatment inhibited CAT activity in all constructs, even when the putative nGRE was missing. Thus, the mechanisms of negative GR regulation of POMC gene transcription remains contraversial.

Glucocorticoid receptor footprinting, deletion and linker substitution mutagenesis, as well as heterologous promoter fusion, have identified a series of receptor binding sequences residing within or near a variety of genes (Karin et al., 1984; Renkawitz et al., 1984; Moore et al, 1985; Denesche et al., 1987; Jantzen et al., 1987). Two distinct classes of DNA sequence elements recognize this single protein species and specify the nature of the regulatory response, a positive regulatory sequence known as GRE and a negative regulatory sequence nGRE. However, comparison of the nucleotide sequence recognized by GR in POMC, prolactin and a subunit of glycoprotein hormone genes does not allow one to derive a clear consensus sequence (Beato, 1989). The differences in the nucleotide sequence of these cis-acting elements in the POMC promoter may be important in mediating positive or negative hormonal effects.

The negative regulation of the a-glycoprotein gene by glucocorticoids has been shown to occur through interference with a cAMP responsive enhancer (Akerblou et al., 1988). Through DNase I and methylation protection analysis, Mellon and colleagues (1988) demonstrate the GR complex may prevent the binding or function of the CRH-binding proteins by overlapping the CREs. Interestingly, it is not the nucleotide sequence of the a-subunit promoter that determines transcriptional efficiency. Rather the particular localization of the binding site in the context of the regulatory region appears essential for the functional interaction among steroid hormones and other essential transcription factors.

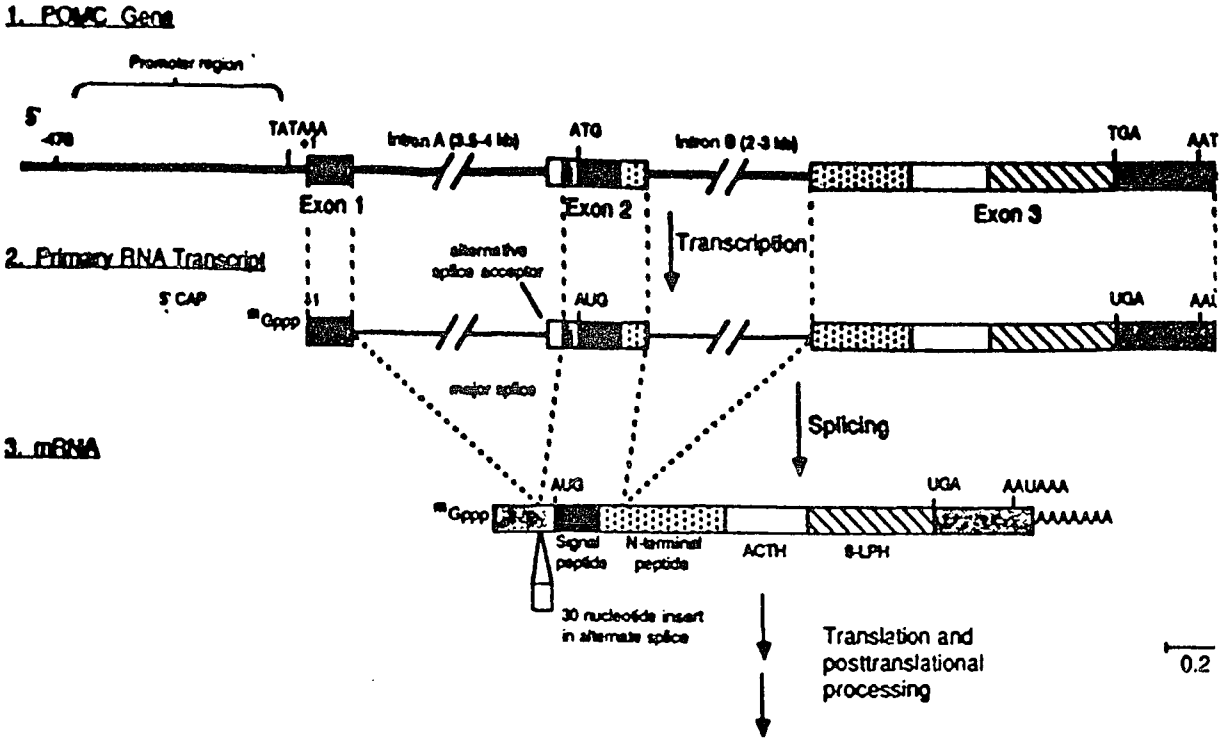

Figure 1. Rat POMC gene structure. This schematically shows the POMC gene, primary RNA transcript, and mature mRNA as expressed in the anterior lobe of the pituitary. Figure from Lundblad and Roberts (1988).

TABLE I. Electrical and pharmacological properties of the three types of vertebrate calcium channels in chick DRG neurons[19,24]

Channel type	T	N	L
Single-channel conductance (110 Ba)	~ 8 pS	~ 13 pS	~ 25 pS
Single-channel kinetics	Late opening brief burst, inactivation	Long burst, inactivation	Almost no inactivation
Relative conductance	$Ba^{2+} = Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$
Inorganic ion block	$Ni^{2+} > Cd^{2+}$	$Cd^{2+} > Ni^{2+}$	$Cd^{2+} > Ni^{2+}$
ω-CgTx via block	Weak, reversible	Persistent	Persistent
Dihydropyridine sensitive?	Resistant	Resistant	Sensitive
Activation range[a] (for 10 Ca)	Positive to −70 mV	Positive to −20 mV	Positive to −10 mV[a]
Inactivation range (for 10 Ca)	−100 to −60 mV	−120 to −30 mV	−60 to −10 mV
Inactivation rate[b] (0 mV, 10 Ca or 10 Ba)	Rapid (tau ~20–50 ms)	Moderate[b] (tau ~50–80 ms)	Very slow (tau > 500 ms)

[a] Activation range may be considerably more negative in sympathetic neurons, chromaffin cells and other cell types. [b] Rate of inactivation of N-type channels can be much slower in sympathetic neurons. Inactivation rates may also be different in the absence of the exogenous cytoplasmic Ca^{2+} buffers introduced by whole cell recordings.

From Tsien et al., 1988, Table 1.

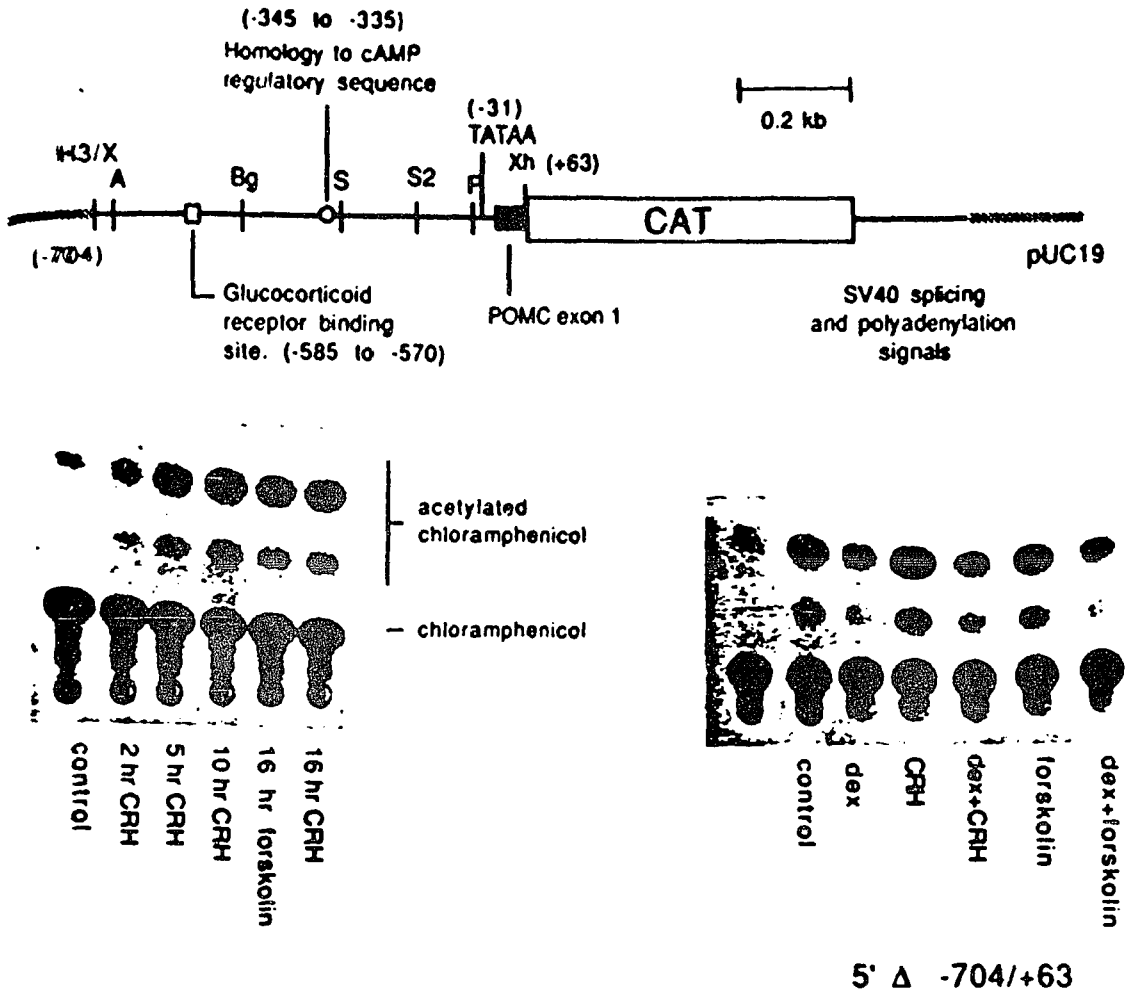

FIGURE 2. Hormonal responsiveness of a POMC-CAT fusion gene in AtT20 cells. Rat POMC 5' flanking sequences (−704 to +63) including the transcription initiation site, POMC promoter and a portion of exon 1 were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene in a pUC19-derived plasmid and transfected into AtT20 cells for transient expression. 48 hours posttransfection, CRH (10 nM) or forskolin (10 μM) with or without 100 nM dexamethasone was added in fresh DMEM. Cells were harvested and cytoplasmic extracts were assayed for the ability to acetylate ^{14}C-chloramphenicol. Products were then separated by TLC and the plate exposed to x-ray film.

From Roberts et al., 1987, Figure 4.

Chapter 2

Hormonal Regulation of POMC Transcription and RNA Processing in AtT20 Cells.

Abstract

Proopiomelanocortin (POMC) expressing AtT-20 D16/16 tumor cells were utilized to characterize the nuclear processing of POMC heteronuclear RNA (hnRNA) and the transcriptional responses of the POMC gene to various hormonal and pharmacological stimuli. Using intron/exon junctional RNA probes in a solution hybridization/nuclease protection assay (SH/NP), it was determined that the nuclear processing of POMC primary transcript involves first the removal of the 3'-most intron (intron B) and then the removal of the last 5'-intron (intron A) to generate mature mRNA. Since POMC hnRNA levels paralleled changes in POMC gene transcription measured in a nuclear transcription run-on assay under various hormonal and pharmacological conditions, we utilized the quantitative SH/NP assay for a more extensive analysis of factors that modulate POMC gene expression. Corticotropin releasing hormone (CRH) and other cAMP-elevating agents gave time-dependent, 2- to 3-fold increases in POMC hnRNA relative to controls. The synthetic glucocorticoid dexamethasone rapidly inhibited hnRNA levels and attenuated the subsequent ability of CRH to stimulate POMC gene expression. Phorbol esters had no effect on basal hnRNA levels and did not potentiate the effects of CRH. In summary, the measurement of primary transcript and its faithful representation of transcription rate as measured by nuclear run-on assay has allowed for a more detailed analysis of factors which modulate POMC gene expression in AtT20 cells. Our data also clearly shows the absence of short-term effects of protein kinase C activators and the stimulatory effects of protein kinase A activators on POMC gene transcription.

Introduction

The proopiomelanocortin (POMC) gene encodes a protein product precursor to a variety of important neuropeptide modulators, including adrenocorticotropin (ACTH), b-endorphin and the melanotropins (a-, b- and g-MSH). Its main site of expression in the mammal is in the two cell types of the pituitary: the anterior lobe corticotrope and the intermediate lobe melanotrope (Krieger et al., 1980). The expression of the POMC gene in the corticotrope cells of the mammalian pars distalis is under multihormonal control. It is postively regulated by several peptide hormones, including corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), both of which are synthesized in hypothalamic neurons and released into the hypophysial portal blood to exert their stimulatory effects on the anterior pituitary. In a classic feedback inhibition loop, the adrenal corticosteroids negatively regulate POMC gene expression and peptide release by inhibitory effects on the hypothalamus and pituitary (see review Lundblad & Roberts,1988).

Upregulation of POMC gene expression in the anterior pituitary by CRH is mediated in part by the well characterized cAMP second messenger pathway (Aguilera et al., 1983; Labrie et al., 1982a,b; Litvin et al., 1984; Miyazaki et al., 1984; Reisine et al., 1985, 1986). Acute treatment with CRH and pharmacological agents which bypass the CRH membrane receptor to increase cytosolic cAMP levels induce the release of POMC derived peptides from corticotropes *in vivo* (Bruhn et al., 1984) and *in vitro* (Loeffler et al., 1985; Dave et al., 1987). Similar treatments have also been shown to exert stimulatory effects on POMC mRNA levels, at least in part due to transcriptional activation, in anterior pituitary cells *in vivo* (Autelitano et al., 1990) and *in vitro* (Eberwine et al., 1987; Gagner & Drouin, 1985), as assessed by nuclear transcription run-on assays. The finding that CRH and other cAMP elevating agents also stimulate POMC peptide release and mRNA levels in AtT20 cells, a homogeneous mouse corticotrope tumor cell line, suggests that these effects act directly on the POMC cell and not through some paracrine factor.

Glucocorticoids, which by themselves have no immediate effect on basal POMC-derived peptide release, elicit an inhibitory effect on POMC mRNA levels which is preceded by a rapid inhibitory effect on POMC gene transcription in anterior pituitary cells both *in vivo* (Birnberg et al., 1983; Eberwine & Roberts, 1984; Roberts et al., 1979) and *in vitro* (Gagner & Drouin, 1985; Eberwine et al., 1987). In primary cultures pretreated with the synthetic glucocorticoid dexamethasone (Dex) followed by short-term CRH and Dex cotreatment, POMC gene transcription was found to be intermediate between control levels

and those of CRH alone (Eberwine et al., 1987; Gagner & Drouin, 1985, 1987), corresponding temporally with Dex inhibition of CRH-stimulated POMC peptide secretion (Eberwine et al., 1987; Gilles and Lowry, 1978). Conversely, when CRH is added prior to Dex treatment, Dex fails to inhibit POMC gene transcription at a time when CRH has maximally upregulated the gene. These results suggest that glucocorticoids may interfere with CRH-stimulated signal transduction or the more distal CRH-activated transcriptional machinery in the nucleus in addition to their direct effects on the POMC gene.

The hypothalamic neuropeptide AVP is another positive regulator of POMC gene expression in the anterior pituitary. Unlike CRH, AVP is a weak ACTH secretegogue *in vivo* (MacCann & Fruit, 1957; Yasuda et al, 1978) and *in vitro* (Fleisher & Vale, 1968, Buckingham & Hodges, 1977; Vale & Rivier, 1977) and does not activate the cAMP second messenger pathway. Rather, AVP acts in concert with CRH to modulate POMC gene expression by increasing intracellular calcium and activating protein kinase C (PKC) via stimulation of inositol phospholipid turnover (Abou-Samra et al., 1986; Negro-Vila, 1985; Raymond et al., 1985; Todd & Lightman, 1987). Acute treatment of cultured anterior pituitary cells with AVP markedly potentiates CRH-stimulated secretion of POMC-derived peptides with a similar stimulatory effect on cAMP content (Abou-Samra et al, 1987; Castro et al., 1989; Heisler, 1984; Phillips & Jaken, 1983; Murakami et al., 1984). Activating PKC with tumor-promoting phorbol esters or synthetic diacylglycerol mimics the secretory effect of AVP in primary corticotrophs (Abou-Samra, 1986; 1987), melanotrophs (Loeffler et al., 1989) and AtT20 cells (Heisler et al., 1984; Vyas et al., 1990).

It is not clear whether the biosynthesis of POMC is regulated by cAMP-independent agents at the transcriptional level in a manner that parallels secretion. Levin et al. (1989) demonstrated that although AVP rapidly induces POMC-derived peptide secretion in primary anterior pituitary cultures, it does not regulate basal levels or potentiate CRH-stimulated production of POMC primary transcript as measured by a solution hybridization nuclease/protection assay. Rather, the regulatory effects mediated by AVP may be exerted on mechanisms distal to POMC gene transcription. These results agree with other studies which have demonstratd that PKC activators evoke only slight effects on POMC cytoplasmic mRNA accumulation in primary cultures (Wand et al., 1988; Suda et al., 1989) and in AtT20 cells (Thiele & Eipper, 1990). Therefore, it is unclear whether the second messenger phosphoinositide triphosphate and PKC activation are important in mediating rapid changes in POMC gene expression via transcriptional activation.

As outlined above, previous work characterizing the mechanisms of the hormonal modulation of POMC mRNA levels have been performed in the whole animal or in primary pituitary cultures, both mixed populations of cells. The established AtT20 cell line subclone, D16/16, is an excellent model system for analyzing polypeptide and steroid hormone regulation of POMC gene transcription because it represents a uniform population of corticotropes, retaining characteristics of their *in vivo* past (Sabol, 1980). To date, AtT20 cells have only been used for long term studies measuring mRNA level changes in the cytoplasm, where it is impossible to rule out any effects of hormonal treatment on mRNA stability. The goal of this study is to characterize the rapid regulation of POMC gene transcription by neurohormones in cultured AtT20 D16/16 cells, and to examine the signal transmission linking cell-surface activation by ACTH secretagogues to genomic POMC transcriptional events. Neurohormone regulation of genomic POMC transcriptional events will be measured using two methods: an *in vitro* nuclear transcription run-on assay and a solution hybridization/nuclease protection (SH/NP) assay. The *in vitro* transcription run-on assay accurately measures the rate of gene transcription, but is technically difficult, costly and labor intensive. The SH/NP assay indirectly measures transcription by quantitating the level of nuclear RNA primary transcript, a function of both the rate of transcription and RNA processing to mature mRNA. It is a much simpler and more rapid method, albeit more indirect. In this study, the SH/NP assay was used to measure rapid changes in the level of POMC primary transcript in AtT20 cells. Similar treatments were also tested for their effect on POMC gene transcription by the more difficult nuclear transcription run-on assay in order to compare changes in transcription and primary transcript accumulation.

Methods

Cell culture techniques. Mouse AtT-20 D16/16 tumor cells were cultured in Dulbecco's Modified Eagle medium (DME) supplemented with 10% horse serum and 5% fetal calf serum. Cells were plated in 35-mm diameter culture dishes (Corning) at an initial density of 1.5 x 10^5 cells per plate and were grown in a 5% CO_2 humidified incubator at 37°C. Cells were used for experiments 4-5 days after subculturing (60-80% confluency).

Experimental protocol and RNA isolation. One hour prior to the onset of an experiment, cell medium was removed and replaced with serum-free DME and the cells were returned to the incubator. Rat CRH (Peninsula laboratory, Inc.) was dissolved in 10mM HCl with 0.1% ascorbic acid at a stock concentration of 10mM. Dexamethasone (Sigma) was diluted in ethanol as a 10mM stock. Phorbol esters (Calbiochem) and 3-isobuty-1-methyl-xanthine (IBMX, Sigma) were each dissolved in DMSO as a 10mM stock. 8-bromo-cAMP (8brcAMP, Sigma) was diluted in sterile water as a 100mM stock. These agents were stored at -80°C, except for dexamethasone which was stored at -20°C and diluted in serum-free DME at the time of the experiment. The final dilution of each of the above agents was added to the cells in 1% or less of the total culture volume (2 ml).

At the end of the incubation period, medium was removed and the monolayer was rinsed once with ice-cold 1x phosphate buffered saline (pH 7.4). Cells were lysed by the addition of 0.5 ml/culture of a 0.3M sucrose lysis buffer (10mM Tris-HCl, pH 8, 3mM $CaCl_2$, 2mM $MgCl_2$, 0.5mM DTT, 0.15% Triton), and the lysate was layered over a 0.4M sucrose/Triton cushion. Samples were centrifuged at 600 x g for 10 min at 4°C to pellet nuclei, separating nuclear and cytoplasmic RNA fractions. 0.45 ml of the supernatant containing the cytoplasmic RNA was removed and incubated with 50µl 10xSET buffer (1xSET = 1% SDS, 5mM EDTA, 10mM Tris-Cl, pH 8.0) and 5µl Proteinase K (10mg/ml) at 37°C for 15 min. The cytoplasmic RNA was then purified by extraction with phenol/chloroform and precipitated with 0.1 volume 3M sodium acetate in 2.5 volumes of 100% ethanol.

To purify nuclear RNA, the nuclear pellet was resuspended in 0.5 ml of the 0.3M sucrose lysis buffer and centrifuged at 600 x g for 10 min. The pellet was resuspended in 100 ul DNase buffer (50mM Tris-Cl pH 8, 5mM $MgCl_2$, 0.1mM EDTA) and digested with 30U RNase-free DNase I (Worthington Biochemical Corp.) for 5 min at 37°C, followed by the addition of 0.5 ml 1xSET and 5 µl Proteinase K and incubation at 45°C for 30 min. 200ul

of the sample was removed for DNA quantitation with a diphenylamine assay (see below). The remaining sample was extracted with phenol/chloroform and precipitated in ethanol as described above.

DNA quantitation. DNA was quantitated following the method of Burton et al. (1956). In brief, 0.2 ml 1N perchloric acid was added to 200 ul of nuclear sample and incubated for 20 min at 68°C. The samples were cooled slowly to room temperature, and 0.4 ml of a chromogenic agent (1.5% diphenylamine dissolved in 97% glacial acetic acid, 1.5% sulfuric acid, 16mg/ml acetaldehyde) was added, vortexed and incubated at 30°C for 8-10 hrs. The amount of DNA in the samples was quantitated by measuring absorbance at 600nm in a spectrophotometer. The sample DNA was expressed in micrograms relative to a standard curve generated with known amounts of salmon sperm DNA.

Solution hybridization/Nuclease Protection assay. POMC nuclear and cytoplasmic RNA transcripts were quantitated using the RNase A/T1 protection assay method previously described (Blum, 1989). [^{32}P]-labeled genomic antisense RNA probes (pMex1, pMex2 or pMex3) spanning an intron-exon portion of the mouse POMC gene (Figure 1) were synthesized to a specific activity of $1x10^9$ cpm/ug. *In vitro* synthesized POMC sense RNA was used to generate a standard curve in the range of 5-1000 pg for quantitation of samples. RNA samples and standards were hybridized to RNA probe as previously described (Levin et al., 1989). Following hybridization, samples were digested with 0.3 ml RNase buffer (40mg/ml RNase A (Sigma), 3mg/ml RNase T1 (Sigma), 10mM Tris-Cl pH 8, 5mM EDTA, 300mM NaCl) for 60 min at 30°C. The protected RNA:RNA hybrids were then incubated with 10 ul 10% SDS and 5 ul Proteinase K (10mg/ml) at 37°C for 15 min, phenol/ chloroform extracted, precipitated in ethanol and analyzed on a polyacrylamide gel. The gels were exposed to X-ray film following which radioactive bands corresponding in size to nuclear POMC primary transcript, nuclear mRNA and cytoplasmic mRNA were excised from the gel and counted in a liquid scintillation counter. The radioactivity in protected POMC RNA:RNA hybrids was compared against the radioactivity in protected bands of a standard curve generated with picograms (0-1000 pg) of (+) sense POMC RNA (Fig. 1C). Linear regression analysis converted the radioactivity in sample RNA into picograms of protected standard.

Nuclear Transcription Run-on Assay. Transcription run-on assays using AtT20 cells were performed as previously described (Blum, 1989). Briefly, intact nuclei are isolated from homogenates of Triton-solubilized cells as described above and incubated in

the presence of [^{32}P]-labeled UTP. RNA transcripts initiated by RNA polymerase II complexes *in vivo* are elongated *in vitro* without transcription reinitiation. Nascent labeled RNA transcripts were hybridized to a 924 base pair (bp) mouse POMC cDNA Hind III/ EcoR I fragment (MIKI). Trace amounts of [^{3}H]-labeled MIKI sense RNA (approx. 4000 cpm) were included in the hybridization reaction to correct for hybridization efficiency.

Statistical analysis. Each experimental condition was performed in triplicate and expressed as a mean value ± standard deviation. The data was evaluated by a one factor ANOVA and differences with $P<0.05$ were considered significant (Scheffe test).

Results

Nuclear POMC RNA Processing. The structure of the murine POMC gene and its transcriptional RNA products are repesented schematically in Fig. 1A. Exon 1, exon 2 and exon 3 are 96, 155 and 677 bases in length respectively, and both intron A and B are greater than 1.5 kb in length (Notake et al., 1983). A set of genomic POMC fragments were subcloned into pGEM vectors (Promega) and used to develop quantitative RNA assays as previously described (Blum 1989). The antisense RNA probe (pMex3) generated from this intron B/ exon 3-containing subclone hybridizes to two species of RNA in nuclear RNA samples as depicted under the primary RNA transcript: the larger protected RNA:RNA hybrid is 500bp in length, and corresponds to primary transcript. The smaller hybrid represents mature nuclear mRNA and is 338 bp in length. Similar studies were performed with pMex1 (unfilled bar), a POMC 5' flanking/exon 1/intron A containing probe, and pMex2 (stippled bar) an exon 2/intron B containing probe.

Based on the relative molar ratios of the different protected RNA species using all three POMC intron/exon junctional probes in separate experiments (Table 1), it was concluded that intron B was spliced out first followed by intron A, similar to the POMC RNA splicing pattern observed in the rat (Levin et al, 1989). Further AtT20 nuclear RNA protection studies using a mouse POMC intron A/exon 2/intron B antisense RNA probe confirmed the splicing pattern described in Fig. 1A (data not shown). pMex2 and pMex3 probes protect two RNA:RNA hybrids, the longer primary RNA transcript and the shorter mature nuclear mRNA, whereas the pMex1 probe protects both primary transcript and a nuclear RNA processing intermediate (exon 1/intron A/exon 2/exon 3) as a single hybrid. In this paper, the longer nuclear RNA hybrids protected with the pMex2 or pMex3 probes will be described as primary transcript, and the longer hybrid protected with the pMex1 probe will be called heteronuclear RNA (hnRNA). Since there is approximately 7.5 times more hnRNA measured with pMex1 than primary transcript measured with pMex3 or pMex2 on a molar basis (Table 1), we could more easily detect the inhibitory effects of glucocorticoids on POMC gene expression by measuring hnRNA, and, therefore, we have used pMex1 probes in the majority of our protection assays.

A representative audioradiogram of pMex1-protected nuclear RNA is shown in Fig. 1B. The first three lanes are 10nM CRH treatment for 60 min and the second three lanes are controls. In cytoplasmic RNA samples, each POMC antisense RNA probe protects a single hybrid, representing cytoplasmic POMC mRNA, which is identical in length to the

corresponding nuclear mRNA. The level of AtT20 DNA was measured in an aliquot of nuclear extract and levels of RNA were reported as picrograms RNA per microgram DNA recovered from 1/3 of the cells grown in a 35-mm culture well, as determined by a diphenylamine colorimetric assay (see methods). The level of control POMC primary transcript or hnRNA did not change significantly in any of these experiments, ranging from 12-18.5 pg/ug DNA using the pMex1 antisense RNA probe, and 5-9 pg/ug DNA using pMex3. It should be noted that although primary transcript or hnRNA levels changed rapidly under certain treatment conditions, there were no significant changes in the levels of nuclear or cytoplasmic POMC mature mRNA between treatment groups in these short-term experiments.

The Solution Hybridization/Nuclease Protection Assay vs. Nuclear Run-on Transcription Assay. The direct effects of short-term treatment of AtT20 cells with CRH or the synthetic glucocorticoid dexamethasone (Dex) were measured by a nuclear transcription run-on assay (Fig. 2A). Stimulation of AtT20 cells for 60 min with 10nM CRH resulted in a 2-fold increase in POMC gene transcription over untreated controls. Dex inhibited the level of POMC gene transcription in AtT20 cells to 50% of controls at 60 min. These results are similar to, but not quite as robust as previously published data measuring the effects of CRH and Dex on POMC gene transcription in cultured rat anterior pituitary cells (Eberwine et al., 1987; Gagner & Drouin, 1985).

To determine whether these rapid changes in POMC gene transcription were paralleled by changes in the levels of primary transcript and processing intermediate, we analyzed the short-term effects of CRH and Dex on AtT20 nuclear RNA using either pMex1 or pMex3 antisense RNA probes in a SH/NP assay. 60 min CRH (10nM) treatment produced a 2.4-fold stimulation of POMC primary transcript (Fig. 2B) or a 2.2-fold elevation of POMC hnRNA levels (Fig. 2C), and 60 min Dex (100nM) treatment decreased primary transcript and hnRNA levels to 60% relative to untreated cultures. Changes in the levels of POMC primary transcript or hnRNA, as measured with the pMex3 or pMex1 probes respectively, were not significantly different eventhough the absolute levels of RNA hybrids varied with treatment. These rapidly induced changes in primary transcript and processing intermediate levels parallel the results obtained with the transcription run-on assay in Fig. 2A.

The acute effects of CRH and cAMP-elevating agents on POMC gene expression. Positive regulation of POMC gene expression by CRH involves the cAMP-dependent second messenger pathway (Reisine & Guild, 1985; Aguilera et al., 1983;

Perrin et al., 1986). To determine whether pharmacological elevation of intracellular cAMP in AtT20 cells has a direct effect on POMC gene transcription, the response to the cAMP analogue 8brcAMP was measured by a nuclear transcription run-on assay (Fig. 3A). Treating AtT20 cells with 1mM 8brcAMP resulted in a 2.8-fold increase in POMC transcription at 30 min which was still maintained at 60 min. In a SH/NP assay (Fig. 3B), treatment with 8brcAMP gave similar increases in POMC primary transcript. The effect of 8brcAMP on POMC gene transcription and hnRNA levels parallel the results described above with CRH (Fig. 2A).

The short-term effects of 10nM CRH treatment on the accumulation of POMC hnRNA levels in AtT20 cells was analyzed at various time periods (Fig 4A). CRH treatment significantly and maximally induced hnRNA levels 3.0-fold over controls after 30 min, and the levels remained elevated after 60 and 120 min CRH treatments. Agents which increase intracellular cAMP levels mimicked these rapid effects of CRH on POMC gene expression. The adenylate cyclase stimulating diterpene forskolin (10mM) induced hnRNA levels in AtT20 cells to similar magnitude and with a similar time-course as CRH (Fig. 4B). POMC hnRNA levels increased significantly relative to ethanol-treated controls after 15 min incubation with forskolin, with maximal 2.5-fold induction at 60 min and sustained elevation at 60 and 120 min. The cAMP analog 8brcAMP (1mM) or the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX, 0.5mM), also rapidly increased the accumulation of hnRNA after 15 min and 60min (Fig 4C). Cotreating AtT20 cultures with 8brcAMP and IBMX resulted in a slightly greater induction than treatment with either agent alone (Fig 4C).

Negative regulation by glucocorticoids. The short-term effects of glucocorticoids on basal and CRH-regulated POMC hnRNA accumulation in AtT20 cells were examined. The synthetic glucocorticoid dexamethasone (Dex,100nM) rapidly and significantly decreased POMC hnRNA levels over ethanol-treated controls in a time-dependent manner (Fig. 5). A significant reduction in the accumulation of hnRNA was observed at 45 min and was further reduced to almost 50% of control levels at 60 min. Similar inhibitory effects occured with 10nM Dex at these time points (data not shown). These results agree with earlier reports of rapid down-regulation on POMC gene transcription by dexamethasone and corticosterone in dispersed anterior pituitary cells in culture as measured by a nuclear run-on transcription assay (Birnberg et al, 1983; Eberwine et al., 1984).

To determine whether interactions between glucocorticoids and CRH treatment occured at the transcriptional level, AtT20 cells were pretreated with either CRH or Dex, and then subsequently challenged with both hormones (Fig. 5). Incubation with 100nM Dex for 15 or 30 min, followed by cotreatment with 10nM CRH and Dex for an additional 30 min, resulted in a 50% reduction in hnRNA levels relative to the 2.8-fold induction with 30 min CRH incubation alone. 100nM Dex pretreatment also rapidly attenuated subsequent 30 and 60 min induction of POMC hnRNA levels by 8brcAMP (data not shown). Conversely, pretreating cells with 10nM CRH for 15 min with subsequent CRH and 100nM Dex treatement for 30 min did not result in an attenuation of induction relative to CRH treatment alone for 45 min (Fig. 5).

Protein kinase C activity and POMC gene expression. Possible involvment of the PKC-dependent second messenger pathway in regulating rapid changes in POMC gene transcription was also studied in AtT20 cells. The PKC activating phorbol ester, phorbol 12-myristate 13-acetate (PMA), has no dose-dependent effect on POMC hnRNA levels after 60 min, eventhough in the same experiment IBMX (0.5mM) was able to induce a 2.7-fold effect (Fig. 6A). The more soluble phorbol ester, phorbol 12,13-dibutyrate (1μM), also had no significant effect at 60 min (data not shown). Cotreatment of AtT20 cultures for 60 min with 10nM CRH and 10nM PMA did not potentiate the induction of CRH on POMC primary transcript, while again, treatment with 10nM PMA alone had no significant effect (Fig. 6B). Further pretreatment of cultures for 5 min with 10nM or 100nM PMA prior to stimulation with CRH in the presence of PMA still did not potentiate the inductional effects of CRH on POMC primary transcript levels (Fig. 6C). These results were corroborated by a transcription run-on assay in which 60 min treatment with 100nM PMA alone had no effect on basal or CRH-stimulated POMC transcription in AtT20 cells (Fig. 6D).

Discussion

Intron/exon junctional probes specific to the mouse POMC gene have been used in a solution hybridization/nuclease protection assay to determine the processing pathway of the primary RNA transcription product in AtT20 cells (Fig. 1A). As previously observed in the rat (Levin et al., 1989), intron B is spliced out first and intron A is spliced out last, suggesting that the splicing pattern is not species specific. Consequently, any probes which contain intron B as part of the protected RNA hybrid will only measure POMC gene primary transcript.

We chose to characterize the RNA processing pathway in order to enable us to select probes which would uniquely identify the POMC primary transcript or the processing intermediate in AtT20 cells in a simple, reproducible, although somewhat indirect assay for modulation of POMC gene transcription. As outlined in Fig. 1A, the steady state levels of primary transcript will be determined by two events: the rate of transcription of the POMC gene, and the rate of processing of the primary transcript by the removal of intron B. Since splicing of the primary transcript is a rapid event, creating a small and labile pool of primary transcript, we reasoned that alterations in levels of primary transcript and processing intermediate should be a fairly accurate reflection of alterations in the transcription rate of the gene. This assumption, however, assumes that there are no substantial hormone mediated changes in the processing of the heteronuclear RNA. Testing this would require RNA pulse labelling analysis of the nuclear RNA species which is extremely difficult since changes in the labeled nucleotide triphosphate pool specific activites probably occur on a time scale slower (0.5-2 hrs) than the nuclear processing events (10-60 min). Thus we have chosen an empirical route, measuring levels of POMC gene transcription using the run-on transcription assay and comparing these results with the measured level of POMC primary transcript and processing intermediate. In experiments using peptide, steriod or pharmacological agents known to regulate POMC gene expression, levels of the POMC primary transcript or hnRNA as measured by the SH/NP assay closely reflected the levels of the POMC gene transcription as measured in transcription run-on assays. We have previously observed similar agreement between transcription rate and primary transcript accumulation in the corticotrope of the rat after comparable manipulations, however, the same clear relationship did not appear to be true for the melanotroph (Autelitano et al., 1990). Thus it appears that in the AtT20 cells, which are derived from mouse corticotropes, measurement of the primary transcript or processing intermediate by the SH/NP assay is a good reflection of the level of

transcription of the POMC gene which will facilitate detailed analysis of the second messenger pathways which modulate POMC gene transcription.

Glucocorticoids negatively regulate POMC gene expression in primary anterior pituitary cultures (Eberwine et al., 1987; Gagner & Drouin, 1985) and *in vivo* (Eberwine & Roberts, 1984). Given the heterogeneous population of cells in the anterior pituitary, it is not clear whether or not glucocorticoids act directly on corticotropes to inhibit POMC gene transcription. Since AtT20 cells are a clonal population of transformed corticotrophs, they are a useful model system for studying the direct negative effects of glucocorticoids on the POMC gene transcriptional machinery. Our studies demonstrate that the synthetic glucocorticoid dexamethasone directly and rapidly inhibits POMC gene transcription and POMC primary transcript and processing intermediate levels in AtT20 cells. The magnitude and duration of this inhibition parallels the effects of glucocorticoids on POMC gene transcription described in anterior pituitary cells *in vivo* (Birnberg et al., 1983; Eberwine et al., 1984). The rapid onset of Dex inhibition (as early as 15 min) argues for the existence of a particular sequence which specifies glucocorticoid inhibitory responsiveness in the promoter region of the POMC gene.

The effects of CRH on POMC gene transcription are mediated in part by the cAMP-dependent second messenger system, as has been shown for long-term CRH-stimulated mRNA accumulation in AtT20 cells (Affolter & Reisine, 1985; Reisine et al, 1985). Pharmacologically elevating intracellular cAMP levels with 8brcAMP, forskolin, or the phosphodiesterase inhibitor IBMX rapidly upregulates POMC gene transcription and thus primary transcript and processing intermediate levels in AtT20 cells. This induction is apparent at 15 min, which is consistent with the rapidity by which CRH stimulates adenylate cyclase activity and accumulation of cAMP in AtT20 cells (Heisler & Reisine, 1984). The effects of CRH and intracellular cAMP elevating agents are also similar in time course and magnitude to those observed in primary cultures of rat anterior pituitary corticotrophs (Gagner & Drouin, 1985; Eberwine et al., 1987). Since AtT20 cells are a homogeneous population of cells, this result suggests that the effects observed in primary culture were not due to paracrine factors from other cell types in the anterior pituitary.

Although the POMC gene does not contain a consensus cyclic AMP responsive element in its promoter region, Roberts and colleagues (1987) have identified two CRH response elements in the rat POMC 5' flanking region through the use of fusion gene constructs in a transient expression system. These functional transcriptional elements are located at -478 to

-320 bp and -320 to -133 bp relative to the transcription start site. The absence of a consensus CRE in the POMC promoter suggests the regulation of POMC gene transcription is possibly quite complex, exemplified by the interactions of glucocorticoids and CRH and their regulation of POMC gene expression. Other investigators have reported that in anterior lobe primary cultures, prior treatment with glucocorticoids decreases the subsequent ability of CRH to stimulate POMC peptide release (Widmaier & Dallman, 1984; Buckingham, 1979; Abou-Samra et al., 1986). Eberwine et al. (1987), also working with primary cultures, showed a similar time-dependent effect of Dex on CRH induced POMC gene transcription. Here we report that AtT20 cells also exhibit this phenomenon, presumably due to glucocorticoid reduction of the effectiveness of CRH or 8brcAMP as stimulants of nuclear signal transduction mechanisms in the corticotroph. This glucocorticoid mediated regulation may involve direct protein-protein interactions between the products of the the proto-oncogenes *c-jun* and *c-fos* and the glucocorticoid receptor, as has been demonstrated for proliferin and collagenase gene expression (Diamond et al., 1990; Yang-Yen et al., 1990), since CRH has been demonstrated to induce *c-fos* and *c-jun* in AtT20 cells (personal communication, J. P. Loeffler & A. L. Boutillier).

PKC-regulated gene expression. Data from several groups have shown effects of PKC activators on the POMC system in AtT20 cells or anterior pituitary cell cultures (see reviews Levin & Roberts, 1991; Lundblad & Roberts, 1988). Phorbol esters can stimulate basal POMC peptide secretion from AtT20 cells and have synergistic stimulatory effects with CRH and cAMP (Phillips and Jaken, 1983). Interestingly, phorbol esters lower cytosolic calcium ion levels in AtT20 cells (Luini et al., 1985), suggesting that PKC does not induce POMC peptide release through the same mechanism as cAMP. Phorbol esters have also been shown to induce increases in POMC mRNA levels in AtT20 cells (Affolter & Reisine, 1985; Reisine et al., 1985; Vyas et al., 1990), but only after many hours of treatment, implying that the acute effects on secretion are true secretory effects and not due to an elevated level of biosynthesis.

In these studies, the phorbol ester PMA had no acute effect on POMC gene transcription or primary transcript levels. In addition, we report in the companion paper (Lorang & Roberts, 1991b) that a chronic 24 hour pretreatment with PMA had no effect on either basal or acute CRH stimulated primary transcript levels. Since acute (<60 min) treatments with phorbol esters has been demonstrated to cause a down-regulation of PKC activity levels in AtT20 cells (Bilezikjian & Vale, 1987), we can conclude that this signal transduction

system is not involved in the early effects on POMC transcription produced by CRH treatment. Indeed, in studies using POMC promoter/reporter gene constructs transfected into AtT20 cells we have not seen any effects of PKC activation on POMC promoter activity (Lundblad et al., manuscript in preparation). These results agree with those presented by Levin and colleagues (1989) in their studies of the effects of a PKC activator on POMC primary transcript in rat anterior pituitary cultures. The effects of CRH on POMC gene expression, however, might not be completely independent of the PKC system. Reisine and colleagues (1984) have shown that inhibition of PKC activity in AtT20 cells blocked the subsequent ability of long-term CRH treatment to elevate cytoplasmic POMC mRNA levels. These earlier studies, however, did not address acute events in POMC gene expression, leaving open the possibility of indirect or mRNA stability effects. Thus the reported long-term effects of PKC activation on cytoplasmic levels of POMC mRNA are probably mediated via changes in mRNA stability/ turnover or another indirect effect on later transcription events.

Expression of the POMC gene is altered by a number of hormones and neurotransmitters in the anterior pituitary, resulting for the most part in parallel changes in the synthesis and release of POMC-derived neuropeptides. Most studies have examined the long-term effects of secretagogues on POMC gene expression, requiring several hours before changes in the levels of POMC mRNA and peptide synthesis levels are detected. These studies cannot effectively examine the second messenger systems activated immediately after the binding of a ligand to its receptor. Since the level of primary transcript and processing intermediate appears to accurately reflect changes in POMC gene transcription, we have been able to perform a more detailed description including proper statistical analysis of the factors that modulate POMC gene expression in AtT20 cells.

From the results obtained in this study, we conclude that CRH, via the cAMP-dependent second messenger pathway and other intracellular mediators (Lorang & Roberts, 1991b), rapidly upregulates POMC gene transcription, resulting in elevated levels of the newly synthesized POMC primary transcript and processing intermediate. A rise in POMC mRNA levels in the cytoplasm subsequently can be detected over a period of several hours which has been shown to be a result of efficient splicing of the highly transitory nuclear primary transcript.

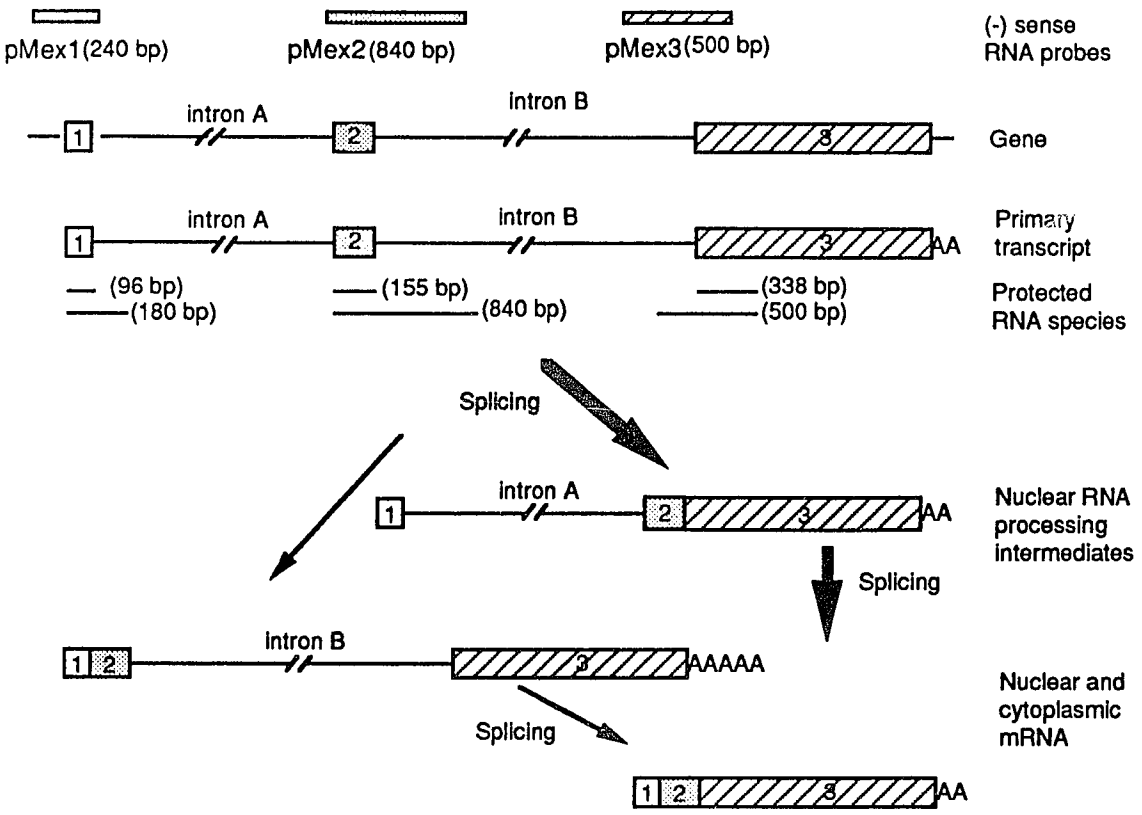

Fig. 1A. A schematic representaion of the mouse POMC gene and processing of the nuclear primary transcript into mature mRNA. Exon 1 is 96 bp, exon 2 is 155 bp, exon 3 is 900 bp, and intron A and B are greater than 1.5 kb each (Notake et al, 1983). Exons are indicated by rectangles labelled 1,2 and 3. The thin rectangles above the POMC gene represent the various POMC intron/exon junctional antisense RNA probes used in protection assays. pMex1 (240 bp, unfilled box) contains a portion of the 5' flanking sequence, exon 1 and 84 bp of intron A. It protects two nuclear RNA:RNA species in the nucleus: a portion of the nuclear mRNA (96 bp) and a portion of the primary RNA transcript and processing intermediate (180 bp). pMex2 (840 bp, stippled box) contains a portion of intron B and 155 bp of exon 2. pMex3 (500 bp, striped box) contains 162 bp of intron B and 338 bp of exon 3. Both pMex2 and pMex3 protect 2 RNA:RNA species: a portion of the nuclear mRNA (96 bp) and a portion of the primary transcript, but not the nuclear RNA processing intermediate since intron B is spliced out first.

Probe	protected fragment (bp)	pg RNA/ ug DNA	fmoles RNA/ ug DNA
pMex1	180	17.8	0.30
pMex2	840	11.0	0.04
pMex3	500	7.5	0.045

Table 1. Sizes and quantities of POMC 1° transcript or hnRNA species protected with various intron/exon junctional antisense RNA probes.

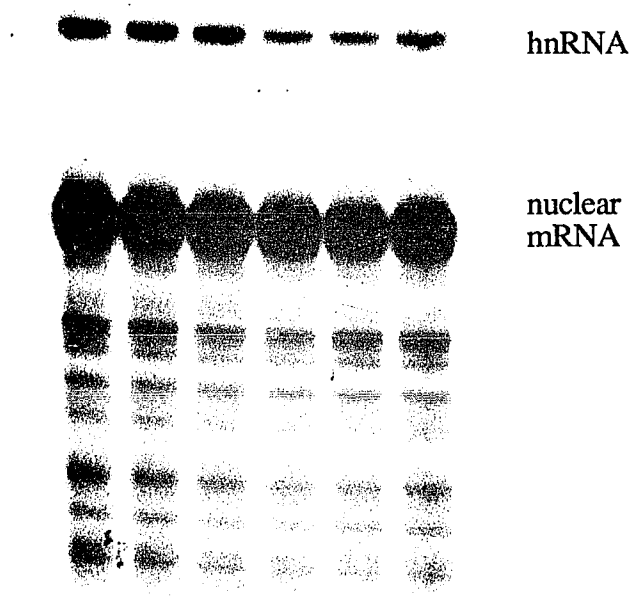

Figure 1B

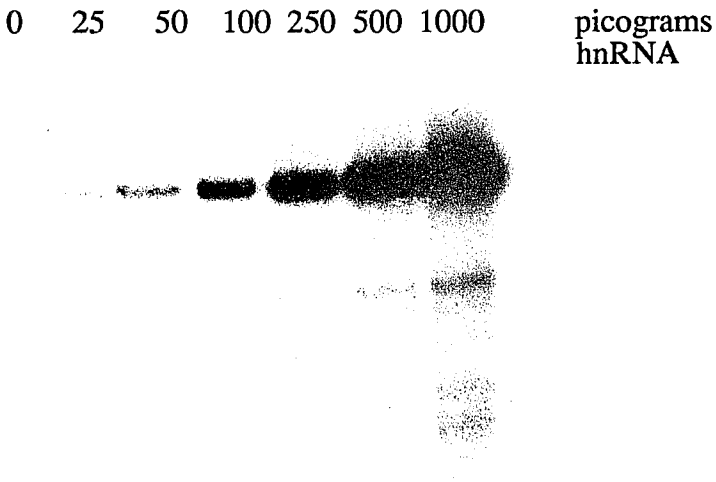

Figure 1C

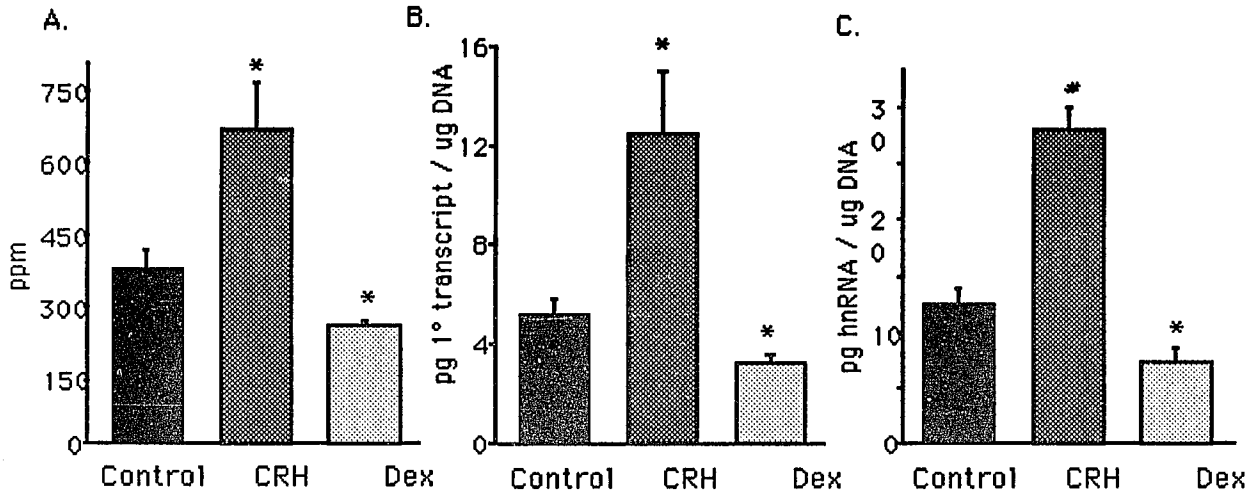

Fig. 2. Analysis of POMC gene transcription in AtT20 D16/16 cultures treated with 10nM CRH or 100nM Dex for 60 min. (A) The rate of POMC gene transcription was measured by a nuclear transcription run-on assay. The data is expressed as the ratio of the specific cpm bound to the POMC gene and the total input cpm (parts per million, ppm) (see Eberwine et al., 1987 fordetails). (B) The level of POMC 1° transcript in cultures receiving identical treatment conditions as in (A) was quantitated by a SH/NP assay using a pMex3 antisense RNA probe and (C) POMC hnRNA (1° transcript and processing intermediate) was measured using a pMex1 RNA probe. *, P>0.05 vs. controls; #, P>0.001 vs. controls.

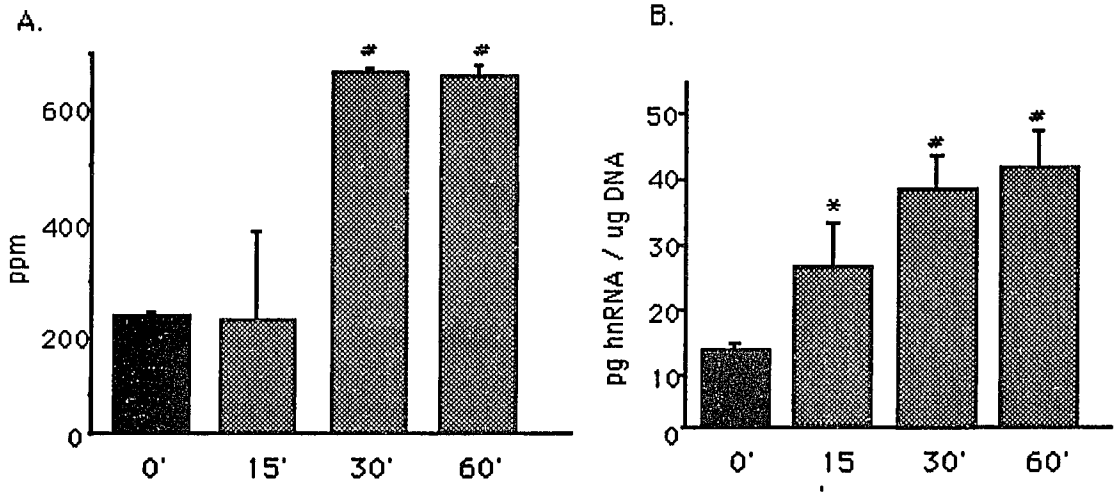

Fig. 3. Time-course effects of 1mM 8BrcAMP on POMC gene transcription in AtT20 D16/16 cells. (A) The short-term effects of 8BrcAMP on POMC gene transcription were measured directly in a nuclear transcription run-on assay. (B) The rapid 8BrcAMP-induced changes in POMC hnRNA levels were quantitated in a solution hybridization nuclease/protection assay. *, P> 0.05 vs. controls; #, P>0.001 vs. controls.

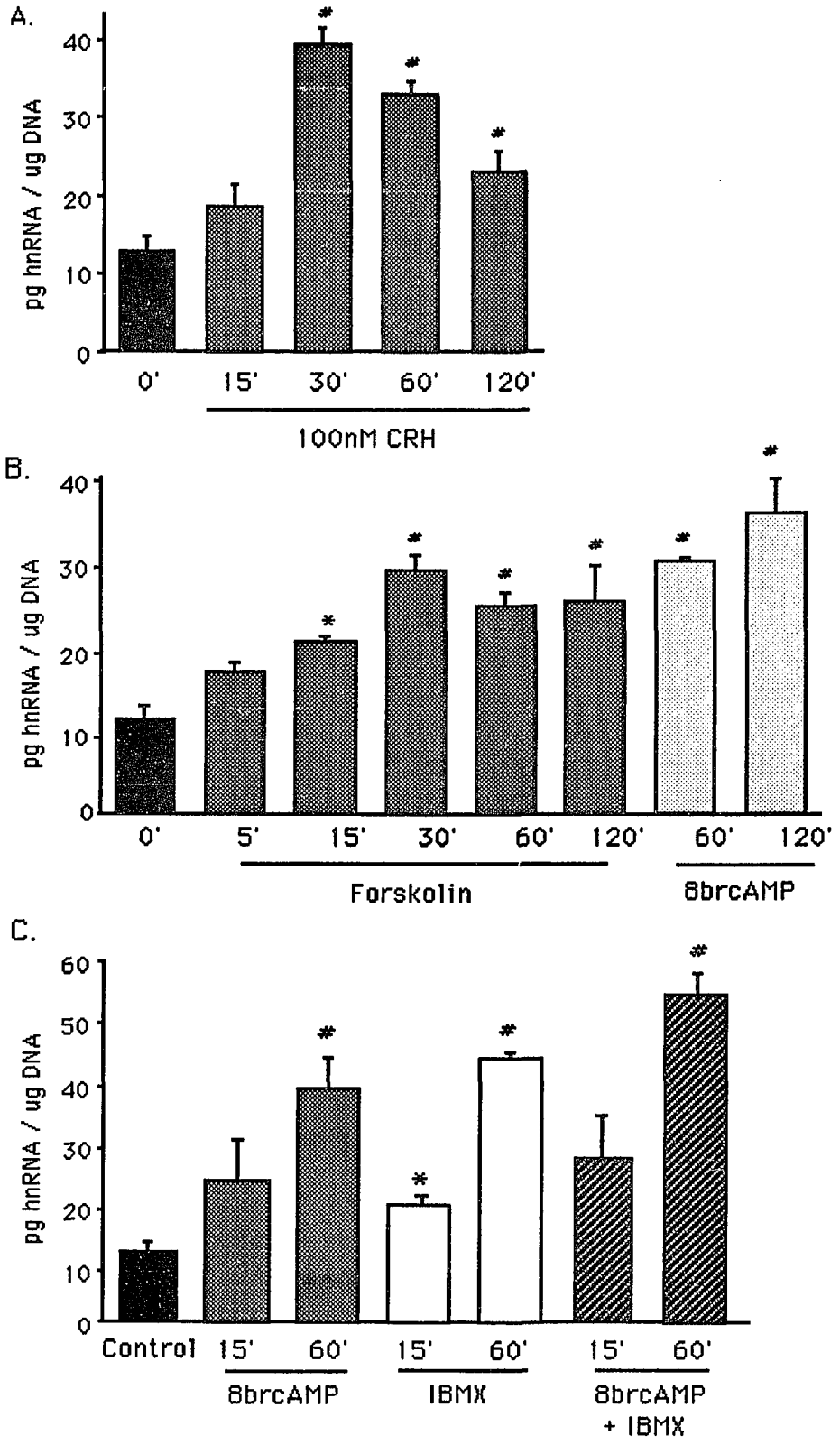

Fig. 4. Time-course effects of cAMP-related agents on nuclear POMC hnRNA accumulation. AtT20 cultures were treated with 10nM rat CRH (A), 10uM forskolin (B) and 1mM 8brcAMP and/or 0.5mM IBMX (C) for the times indicated. *, P>0.05 vs. controls; #, P>0.001 vs. controls.

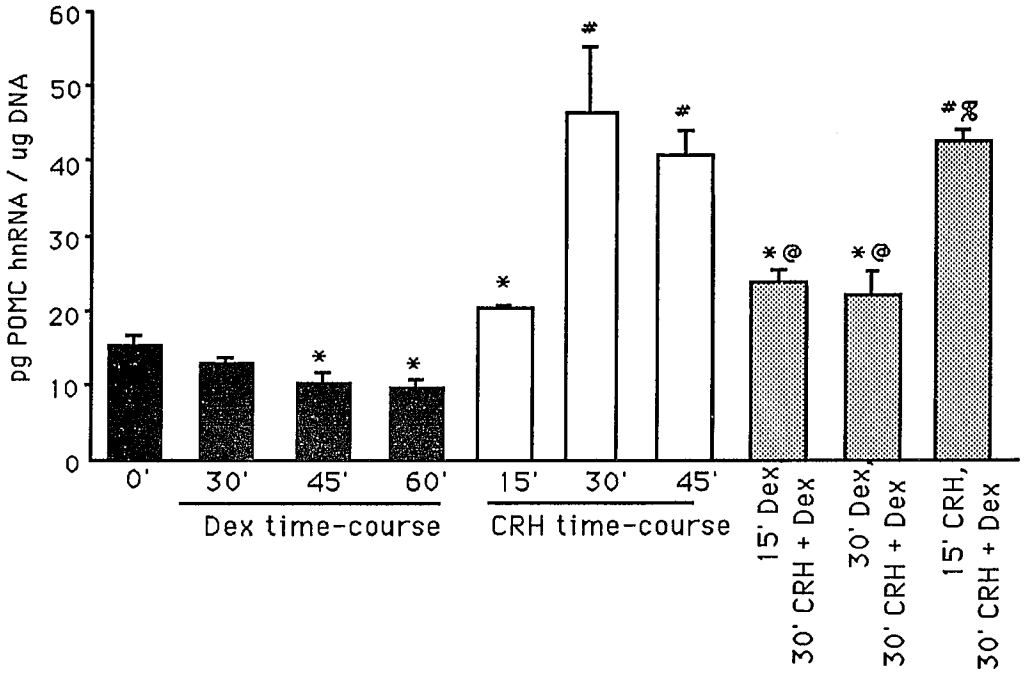

Fig. 5. The inhibitory effects of the synthetic glucocorticoid dexamethasone (Dex) on POMC hnRNA accumulation in AtT20 cells. Treatment with 100nM Dex for 30, 45 or 60 min alone (filled bars) decreases or 10nM CRH for 15, 30 or 45 min (unfilled bars) increases POMC hnRNA levels relative to untreated controls. Pretreatment with Dex for 15 or 30 min attenuates CRH inducibility, whereas pretreatment with 10nM CRH for 15 min has no effect on subsequent combined CRH and Dex (100nM) induction (stippled bars). *, $P<0.05$ vs. vehicle-treated controls; #, $P<0.001$ vs. controls; @, $P<0.05$ vs 30 min CRH treatment; %, $P<0.001$ vs. 45min Dex treatment.

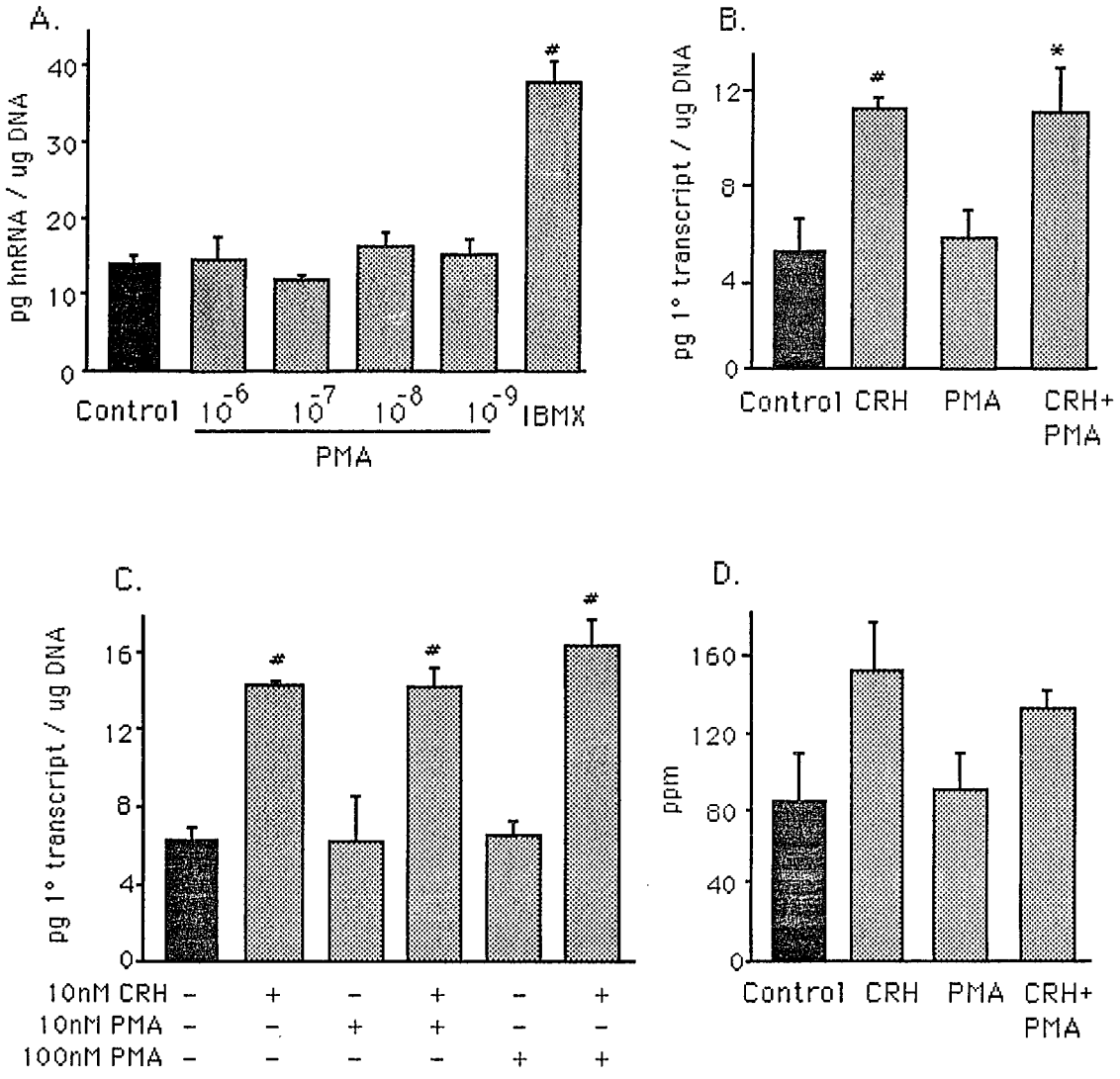

Fig. 6. Effect of short-term treatment of PMA on POMC transcription in AtT20 cells. (A) The level of POMC hnRNA was measured in a PMA dose-response study. (B) The effect of cotreating cultures with 10nM CRH and 100nM PMA on POMC 1° transcript was compared to treatment with each agent alone for 60 min. (C) Cultures received similar treatment conditions as in (B) for only 15 min and PMA concentrations were either 10nM or 100nM as indicated. (D) The effects of 60 min treatment with 10nM CRH and/or 100nM PMA were measured in a nuclear transcription run-on assay. Data is expressed as parts per million (ppm). *, P>0.05 vs. vehicle-treated controls; #, P>0.001 vs. controls.

Chapter 3

Calcium Plays a Major Role in Mediating CRH-Upregulation of POMC Gene Transcription in AtT20 cells.

Abstract

In AtT-20 D16/16 cells it is well documented that Ca^{2+} mobilization is important for modulating basal and CRH-stimulated POMC mRNA levels, POMC-derived peptide synthesis and secretion. The present studies were undertaken to examine the role of Ca^{2+} and cAMP in regulating acute changes in POMC gene expression by corticotropin releasing hormone (CRH) in AtT20 cells. POMC heteronuclear RNA (hnRNA) was quantitated in nuclear RNA samples by a solution hybridization/nuclease protection assay which reflected the changes observed in the rate of POMC gene transcription measured in a transcription run-on assay after acute treatment with Ca^{2+}- and cAMP-elevating secretagogues. Ionomycin or Bay K 8644 (Bay K) treatment for 1 hr increased POMC hnRNA levels 2-fold or greater than controls. Combined CRH and Bay K treatment for 1 hr resulted in an almost 2-fold increase in POMC hnRNA levels over treatment with either agent alone, suggesting an additive interaction between Ca^{2+}- and cAMP-related second messenger pathways. Blocking Ca^{2+} influx with cadmium and cobalt decreased basal hnRNA levels and attenuated subsequent CRH induction eventhough verapamil, ω-Conotoxin GVIA or dihydropyridine antagonists had no effect. CRH continued to increase POMC hnRNA levels in the absence of external Ca^{2+}. The calcium chelator Bapta/AM decreased basal and blocked CRH-stimulated POMC hnRNA accumulation. Together these results demonstrate that elevation of intracellular Ca^{2+} is required for mediating the acute effects of CRH and cAMP on POMC gene transcription, suggesting that Ca^{2+} is the primary second messenger in CRH-regulated POMC gene expression.

Introduction

Studies examining the regulation of Ca^{2+} movement and concentration within a cell have demonstrated that free cytosolic Ca^{2+} is a putative second messenger linking receptor ligand-binding effects at the plasma membrane to changes in neuropeptide gene expression in the nucleus. In pituitary cells, an increase in intracellular Ca^{2+} elicited by any of a variety of methods including depolarization, calcium ionophores, calcium channel agonists or hormones causes an immediate neuropeptide release (Stojilkovic et al., 1988; Won & Orth, 1990a,b; Dave et al., 1987; Heisler, 1985; Tan and Tashjian, 1984). Morever, the expression of several genes has been shown to be regulated by Ca^{2+}, including prolactin (Jackson & Bancroft, 1988; Davis et al., 1988; White et al., 1981), proenkephalin (Nguyen et al., 1990; Kley, 1988), parathyroid hormone (Naveh et al., 1989), insulin (Welsh et al., 1988) and several proto-oncogenes (Morgan & Curran, 1988).

Several studies suggest that the CRH receptor produces its effects on proopiomelanocortin (POMC) peptide secretion in part by increasing cytosolic Ca^{2+} levels. First, transient elevation of intracellular Ca^{2+} has been shown to be a necessary and sufficient signal for the exocytosis of POMC-derived peptides from neurosecretory granules under stimulus-secretion coupling conditions (Imai & Gershengorn, 1986; Luini et al., 1985). Further, CRH causes a rapid rise in cytosolic Ca^{2+} concentration, as measured with the Ca^{2+}-fluorescent dye Quin 2 (Guild & Reisine, 87) and the whole cell patch-clamp technique (Hamill, et al., 1981), in addition to inducing cyclic AMP (cAMP) synthesis and ACTH secretion in AtT20 cells (Luini et al., 1985). The cAMP analog 8-bromo-cAMP (8brcAMP) also produces an increase in Ca^{2+} conductance in patches of AtT20 cells (Luini et al., 1985).

Although the role of Ca^{2+} in POMC-derived peptide secretion is well-characterized, the ability of Ca^{2+} to regulate POMC gene transcription remains to be elucidated. Previous studies have examined the long-term effects of various Ca^{2+} mobilizing agents at the level of POMC mRNA accumulation. Long-term treatment of AtT20 cultures with the calcium ionophore A23187 induces the accumulation of POMC cytoplasmic mRNA relative to controls (Reisine et al., 1985). The specific calcium channel activator Bay K 8644 (Bay K) also increases the level of POMC mRNA (von Dreden et al., 1988) and peptide secretion (Heisler, S., 1985) over vehicle-treated controls. In rat anterior pituitary cultures, A23187 consistently upregulates the rate of POMC gene transcription as measured in a transcription run-on assay (Eberwine et al., 1987). These results led us to investigate the involvement

of Ca^{2+} as a putative second messenger mediating rapid changes in POMC gene transcription in AtT20 cells as a model system.

By measuring the rate of POMC gene transcription in a nuclear transcription run-on assay and levels of POMC hnRNA by solution hybridization/ nuclease protection (SH/NP), we have investigated the acute effects of various pharmacological agents which modulate Ca^{2+} function on POMC gene expression. In this paper we demonstrate that Ca^{2+}, not protein kinase A (PKA) or PKC activation, plays the dominant role in mediating the POMC gene transcriptional response to CRH treatment of AtT20 cells.

Methods

Cell culture techniques. Mouse AtT-20 D16/16 tumor cells were cultured in Dulbecco's Modified Eagle medium (DME) supplemented with 10% horse serum and 5% fetal calf serum. Cells were plated in 35-mm diameter culture dishes (Corning) at an initial density of 1.5 x 10^5 cells/dish and were grown in a 5% CO_2 humidified incubator at 37°C. Cells were used for experiments 4-5 days after subculturing (60-80% confluency).

Experimental procedures and RNA isolation. One hour prior to the onset of an experiment, cell medium was removed and replaced with serum-free DME and the cells were returned to the incubator. In experiments examining the effects of extracellular Ca^{2+} on POMC gene expression, cells were washed 2x with calcium-free DME (Gibco) and incubated in calcium-free DME for <2 hr prior to treatment with hormones or pharmacological agents. Rat CRH (Peninsula Laboratory, Inc.) was dissolved in 0.1% acetic acid as a 10μM stock concentration. Bay K 8644 (Calbiochem), nifedipine (Calbiochem), nitrendipine (Calbiochem) and dexamethasone (Sigma) were dissolved in ethanol. Phorbol 12-myristate 13-acetate (PMA; Calbiochem), BAPTA/am (Molecular Probes), ionomycin (Calbiochem), W13 (Calbiochem) and calmidizolium (Boehringer) were dissolved in DMSO. 8-bromo-cyclic AMP (Sigma) and cadmium (Sigma) were resuspended in sterile water as a 100mM or 1M stock concentration respectively. These agents were stored at -80°C, except for dexamethasone which was stored at -20°C, and cadmium which was stored at -4°C. The final dilution of each of the above agents was added to cells in 1% or less of the total culture volume (2 ml). Cell treatment with hormones and pharmacological agents, nuclear RNA isolation and DNA quantitation were performed as described in the methods section of the previous paper.

Solution Hybridization/Nuclease Protection assay and Nuclear Transcription Run-on Assay. The levels of POMC nuclear and cytoplasmic RNA transcripts or the rate of POMC gene transcription were quantitated as described in the methods section of Chapter 2. [^{32}P]UTP-labeled genomic antisense RNA probes (pMex1 or pMex3) spanning intron-exon portions of the mouse POMC gene were transcribed from subclones contained in the phagemid vector pGem3 (Stratagene) (see figure 1 in Chapter 2) for use in the nuclear SH/NP assays. As a result of the order in which intron A and intron B are spliced out of the POMC primary transcript (see following paper), the pMex3 probe measures POMC primary transcript levels, whereas the pMex1 probe measures both

primary transcript and processing intermediate, which will be referred to as heteronuclear RNA (hnRNA) in this paper.

RIA. Two fractions (1ml) of the cell media were collected and stored at -20°C until thawed on ice and assayed for ACTH by RIA. Details of the double antibody RIA for ACTH have been previosly reported (ref). The ACTH antibody (RSL# 1769) recognized only ACTH (1-39) and ACTH (1-24) of the POMC precursor molecule. The sensitivity of the assay was 1 pg ACTH/ assay tube.

Statistical analysis. Each experimental condition was repeated in triplicate and expressed as a mean value $\pm$ standard deviation. The data was evaluated by a one factor ANOVA and differences with $p < 0.05$ (Scheffe F-test) were considered significant.

Results

Calcium ionophore. To evaluate the effects of elevated intracellular Ca^{2+} without activation of membrane receptors, AtT20 cultures were treated with the calcium ionophore ionomycin (Liu & Hermann, 1987). Ionomycin (1μM) treatment induced the accumulation of POMC primary transcript levels to appromimately 2-fold control levels at 15 min and maintained the elevation for 2 hrs (Fig. 2A) as measured in a SH/NP assay using a pMex3 antisense RNA probe. Cotreating AtT20 cells for 60 min with 1uM ionomycin and 10nM CRH (Fig. 2B) or 1uM ionomycin and 1mM 8brcAMP (Fig. 2C) had an additive effect on POMC primary transcript levels versus treatment with either agent alone. Mature nuclear POMC mRNA levels, under short-term (<120 min) treatment with ionomycin as well as other hormonal or pharmacological agents described in this paper, did not differ significantly from vehicle-treated controls (data not shown).

Calcium channel agonists. Activating L-type voltage-gated calcium channels (VGCC) in AtT20 cells with the dihydropyridine (DHP) agonist Bay K 8644 (Bay K; 1μM), induced the level of POMC primary transcript 3.3-fold over ethanol-treated controls after 60 min, but only 2-fold after 120 min (Fig. 3A). Cotreatment with 1μM Bay K and 10nM CRH for 60min or 120min (Fig. 3A), or cotreatment with 1mM Bay K and 1mM 8brcAMP (Fig. 3B) resulted in an additive effect on primary transcript (CRH) or hnRNA (8brcAMP) accumulation versus treatment with either agent alone. The stimulatory effects of Bay K on POMC primary transcript levels were blocked by the synthetic glucocorticoid dexamethasone (Dex, 100nM) in cells pretreated for 15 min with Dex prior to combined treatment with Bay K and Dex for 60 min (Fig. 3C). Bay K (1μM) treatment alone for 60 min also induced the rate of POMC transcription approximately 2-fold relative to ethanol-treated controls, as measured in a transcription run-on assay in AtT20 cells (Fig 3D). A greater induction of transcription occured in cells coincubated with Bay K and 10nM CRF, but this effect was not significant relative to controls due to the scatter in data points which occur often with run-on assays. The effect of Bay K on POMC gene transcription appears time-dependent, with stimulatory effects detected as early as 15 min, and 2-fold or greater induction at 30 min and 60 min, as measured in a run-on assay (Fig. 3E). The DHP calcium channel blocker nifedipine (NF, 1μM) completely blocks POMC primary transcript accumulation in cells cotreated with Bay K and NF for 60 min (data not shown), demonstrating that the effects of Bay K are specific to L-type VGCC activation in AtT20 cells.

PKC-downregulation. Phorbol ester downregulation of PKC activity in AtT20 cells (Bilezikjian et al., 1987) with 100nM PMA pretreatment for 24 hrs did not block subsequent Bay K or 8brcAMP induction of POMC gene expression in AtT20 cells (Fig. 4). Treatment of PKC-downregulated cells for 60 min with either 1mM 8brcAMP or 1μM Bay K induced the accumulation of POMC primary transcript to levels greater than two-fold higher than PMA-pretreated controls. This level of induction does not differ significantly that observed under PMA-free conditions. Cotreating PMA-down regulated cells with 8brcAMP and Bay K further enhanced the induction of primary transcript as compared to treatment with either agent alone. Again, the stimulatory effects observed in PKC-downregulated AtT20 cells parallel the effects observed with 8brcAMP and Bay K in the absence of PMA treatment.

Calcium channel antagonists. A number of calcium channel antagonists have been shown to block VGCC activity in the pituitary (Armstrong & Kalman, 1987). Our study demonstrates that short-term treatment with the highly specific DHP calcium channel antagonists NF (1μM) or nitrendipine (NTP, 1μM) does not significantly alter basal POMC primary transcript or hnRNA accumulation in AtT20 cells (Table 1). Induction of POMC hnRNA by CRH is not attenuated by 15 min pretreatment with NF or NTP and subsequent costimulation with CRH and either of the two DHP antagonists for 60 min. These results contrast with the short-term inhibitory effects of NF on basal and CRH-stimulated ACTH secretion seen in AtT20 cultures (data not shown) as well as the inhibition mediated with VGCC blockers in rat anterior pituitary cells *in vitro* (Won & Orth, 1990; Dave et al., 1987; Murakami et al., 1985).

The effects of other calcium channel antagonists bearing no structural similarity to the DHPs and demonstrating less selectivity for L-type VGCC were examined. They included the phenylalkylamine verapamil (VP, 10μM) and the 27-amino acid peptide toxin, w-Conotoxin GVIA (conotoxin, 10nM or 100nM) derived from the marine snail *Conus geographica* (McCleskey et al, 1987; Reynolds et al., 1986). The effects of VP or conotoxin on POMC gene expression were identical to those of NF or NTP as previously described. The inability of these VGCC antagonists to block basal or CRH-induced POMC primary transcript or hnRNA levels was confirmed by a series of transcription run-on assays in which NF or conotoxin did not alter the rate of basal or CRH-regulated POMC gene transcription (Table 2).

Cadmium and Cobalt ions. Since the previously described VGCC antagonists did not block CRH-regulated POMC gene expression, we treated AtT20 cells with more general Ca^{2+} channel blockers, the divalent cations cadmium (Cd^{2+}) or cobalt (Co^{2+}). Cd^{2+} and Co^{2+} are potent calcium channel blockers as measured in electrophysiological studies of various cell types (Miller, 1987; Tsien, 1983; Nelson, 1986; Matteson & Armstrong, 1986) and compete with Ca^{2+} for binding intracellular regulatory proteins. It has been demonstrated by Hinkle and colleagues (1987) that the flux of Cd^{2+} through VGCC is the major mechanism of Cd^{2+} uptake by anterior pituitary GH_4C1 cells since treatment with calcium channel blockers protect cells from Cd^{2+} toxicity. Pretreating AtT20 cells with 500μM Cd^{2+} for 15 min blocked the subsequent stimulatory effects of 60 min treatment with 10nM CRH (Fig. 5A) on POMC hnRNA accumulation. Similar effects were seen with 50μM Cd^{2+} (Fig. 5C and data not shown). The stimulatory effects of 60 min treatment with 1mM 8brcAMP were also blocked by pretreatment with 500μM Co^{2+} for 15 min (Fig. 5B). Cd^{2+}-dependent inhibition of CRH-stimulated POMC hnRNA accumulation is prevented in cells pretreated with 1mM NF for 15 min, since NF blocks Cd^{2+} uptake by cells (Fig. 5C). AtT20 cells were treated with 50mM Cd^{2+} (or 1μM NF) for 15 min followed by the addition of 1mM NF (or 50μM Cd^{2+}) for an additional 15 min, after which 10nM CRH was added to the media for 30 min. Cells were also pretreated with either Cd^{2+} or 1μM NF for 15 min prior to 30 min CRH stimulation. NF pretreatment blocked (Fig. 5C) the inhibitory effects of subsequent Cd^{2+} on CRH-regulated POMC gene expression. In the reversed treatment paradigm, the protective effects of NF on CRH inducibility were abolished by initial Cd^{2+} pretreatment.

Extracellular Ca^{2+}. Since it has been demonstrated that removing extracellular calcium is more effective than VGCC blockers at inhibiting the effects of CRH on ACTH secretion (Abou-Samra et al., 1987; Won & Orth, 1990), we examined second messenger-regulated POMC hnRNA accumulation in AtT20 cells incubated in calcium-free DME with added EGTA. Despite deprivation of cells of extracellular Ca^{2+} (calcium-free DME with added 0, 50, 200 or 500mM EGTA) for 2 hrs or less, subsequent addition of 10nM CRH (Fig. 6A, 6B & 7) or 1mM 8brcAMP (Fig. 7) for 60 min rapidly increased the accumulation of POMC primary transcript. Under these calcium-free conditions, both basal and stimulated transcription were signficantly depressed by 31% relative to calcium-supplemented treatment conditions, however, the level of induction obtained with CRH and 8brcAMP with Ca^{2+}-free DME was similar in magnitude to that measured in the presence of external Ca^{2+}. Adding Ca^{2+} back to cells that were previously deprived of external calcium for 2 hrs induced greater than 7-fold POMC primary transcript accumulation relative to calcium-

free controls in the presence or absence of 10nM CRH or Bay K cotreatment for 60 min (Fig. 6B). Increasing the flux of Ca^{2+} through VGCC with the DHP agonist Bay K (1mM) had no effect on POMC primary transcript accumulation in the absence of external Ca^{2+} (Fig. 6A,B) nor did it further enhance the stimulatory effects of 1mM 8brcAMP (Fig. 7).

Calmodulin. AtT20 cells were treated with the calmodulin inhibitors W13 or calmodizolium (CMDZL) in order to determine whether calmodulin activity is an important mediator of the effects of Ca^{2+} on basal and CRH-regulated POMC gene transcription. One hr pretreatment of cells with either W13 (10mM) or CMDZL (1.0 or 5.0μM) followed by addition of 10nM CRH for 30 min did not block basal or CRH-regulated POMC primary transcript accumulation (Fig. 8A). Nor did 12 hr pretreatment of the cells with either 10μM W13 or 1μM CMDZL have any effect on subsequent 60 min 10nM CRH stimulation of POMC primary transcript levels (Fig. 8B).

Intracellular calcium. In order to determine whether levels of intracellular free Ca^{2+} are important for basal and CRH-meditated regulation of POMC gene expression, AtT20 cultures were treated with a membrane-permeable Ca^{2+} chelator, Bapta/AM (Harrison & Bers, 1987; Tsien, 1980), at varying concentrations as indicated in Fig. 9. Four hour cell treatment with 1mM (or 2μM, data not shown) Bapta/AM had no effect on basal or 60 min CRH-stimulated POMC primary transcript levels, whereas 10μM (or 50μM, data not shown) Bapta/AM completely blocked CRH-inducibility and reduced basal levels by greater than 50%.

Discussion

In this study, we demonstrate that calcium-dependent mechanisms are involved in the post-receptor mediation of CRH stimulation of POMC gene transcription in the homogeneous corticotroph population of AtT-20 D16/16 tumor cells. Pharmacological mobilization of Ca^{2+} from extracellular stores via the calcium ionophore ionomycin or the highly selective L-type calcium channel agonist Bay K rapidly stimulates the transcription of the POMC gene and increases the levels of POMC primary transcript and processing intermediate in ATt20 cells. Similar positive transcription effects have been described previously for the prolactin gene in GH3/B6 cells (Laverriere et al., 1989). Studies blocking the entry of extracellular calcium through VGCC suggest that internal Ca^{2+} stores are sufficient for transcriptional activation. In this paper, we demonstrate that inhibiting the rise of intracellular Ca^{2+} from intracellular and/or extracellular stores with the calcium chelator Bapta/AM, blocks CRH or 8brcAMP activated POMC gene transcription, underscoring the importance of Ca^{2+} in the regulation of this event.

In order to determine whether the flux of Ca^{2+} through specific types of calcium channels is necessary for CRH-regulated POMC gene transcription, the effects of specific VGCC antagonists were examined in AtT20 cells. Unlike the calcium channels in brain which appear to be largely dihydropyridine (DHP)-insensitive, VGCC in the pituitary are DHP-sensitive and can therefore be blocked with highly selective DHP anatgonists such as NF and NTP (Fleckenstein, 1983). These antagonists have no effect on CRH stimulated ACTH release rates (Abou-Samra et al., 1987), and in our study, DHP blockers, as well as other VGCC antagonists such as VP and conotoxin, did not block basal or CRH-evoked POMC gene transcription or nuclear POMC primary transcript or hnRNA accumulation. Thus, while flux of Ca^{2+} through VGCC is not necessary for CRH-activated POMC gene transcription, the elevation of intracellular Ca^{2+} by Bay K treatment, which prolongs the opening-time of L-type Ca channels, is sufficient for gene activation. Other studies have shown that basal growth hormone gene transcription in rat anterior lobe cultures (Barinaga et al., 1985), thyroid releasing hormone-induced prolactin gene transcription (Laverriere et al., 1989), and Nerve Growth Factor-stimulated *c-fos* gene expression in PC12 cells (Morgan & Curran, 1986) are insensitive to the presence of DHP antagonists, further implying that basal and polypeptide hormone-stimulated neuropeptide gene transcription are independent of VGCC regulation.

At first glance, our data seem to be inconsistent with previous reported effects of VGCC blockers on POMC gene expression in that Ca^{2+} channel antagonists inhibit basal and CRH-stimulated POMC mRNA levels in rat anterior pituitary cells in culture (Loeffler et al., 1986) and AtT20 cells (von Dreden et al., 1988). In these studies, long-term treatment (>24hrs) was necessary to produce a significant reduction in POMC gene expression. Such chronic treatment with VGCC antagonists may evoke post-transcriptional mechanisms, such as mRNA stability effects, which could not be examined by measuring transcription rate or primary transcript levels. Therefore, in our studies measuring acute changes in POMC gene transcription, the long-term secondary effects accompanying VGCC blockade are minimized.

Multiple calcium channels may be involved in mobilizing Ca^{2+} in corticotropes, offering the cell some flexibility in the way it can modulate the entry of Ca^{2+} and activate POMC gene expression. To block multiple calcium channel types in AtT20 cells and not simply VGCCs as previously described, we used the divalent metal channel anatgonist Cd^{2+} which has been shown to permeate cells primarily through VGCCs (Hinkle et al., 1987) and to compete with Ca^{2+} both at calcium channels (Miller et al., 1987; Tsien, 1983; Nelson, 1986; Suszkiw et al., 1984) and at intracellular binding proteins (Richard et al., 1986). We report here that the calcium channel blocker, NF, prevents the inhibitory effects of Cd^{2+} on POMC gene transcription in AtT20 cells, presumably by blocking Cd^{2+} uptake, as previously described in GH4C1 cells (Hinkle et al., 1987). Cd^{2+}-mediated inhibition of this CRH regulatory event is therefore mediated via an intracellular interaction with Ca^{2+} regulatory proteins, and not as a result of plasma membrane channel blockade. This result is in agreement with the Ca^{2+} -free DME studies, in which CRH inducition of POMC hnRNA levels occured in the absence of extracellular Ca^{2+} .

In the absence of extracellular Ca^{2+}, when Ca^{2+} is presumably mobilized only from internal stores, CRH or 8brcAMP continued to stimulate rapid changes in POMC primary transcript levels with a magnitude of induction similar to that obtained in the presence of external Ca^{2+}. However under these Ca^{2+}-free DME conditions, the overall level of basal POMC primary transcript accumulation was reduced relative to cells incubated in Ca(+) DME, suggesting that Ca^{2+} is necessary for regulating basal POMC gene expression. Interestingly, adding back Ca^{2+} to the media of cells previously incubated in Ca^{2+}-free DME induces the accumulation of POMC primary transcript to levels as high as with CRH treatment alone, further indicating that Ca^{2+} is an important regulator of POMC gene expression. Chelating free intracellular Ca^{2+} with the membrane-soluble EGTA-derived

Bapta/AM depressed basal and blocked CRH-stimulated POMC primary transcript levels in AtT20 cells. In another study, cytosolic Ca^{2+} depletion by preincubation with EGTA also completely inhibited CRH-stimulated cAMP production and ACTH release (Abou-Samra et al., 1987). Overall our studies indicate that external or internal Ca^{2+} sources are capable of elevating free intracellular Ca^{2+} necessary for mediating the stimulatory effects of CRH on POMC gene transcription.

Ca^{2+} alters cellular activity through pathways that involve specific protein kinases, including PKC and the calcium/ calmodulin-dependent kinase (CaM kinase) (Kennedy, 1989). Since cytosolic Ca^{2+} appears important in the regulation of POMC gene transcription in AtT20 cells, we examined whether or not these calcium-dependent kinases coupled the ion flux signal to short-term changes in POMC gene expression. Short- or long-term treatment with W13 or CMDZL was insufficent in blocking the stimulatory effects of CRH on POMC hnRNA accumulation, indicating that the Ca^{2+}-binding protein calmodulin and CaM kinase activation may not be important intermediates in the regulation of POMC gene expression in AtT20 cells. Blocking PKC activity with high PMA concentrations also did not attenuate the induction of POMC primary transcript or hnRNA levels by 8BrcAMP or Bay K. In another study in which AtT20 cells were rendered PKC-deficient (Bilezikjian & Vale, 1987), CRH-stimulated POMC-peptide secretion also remained unaltered, further supporting the idea that PKC does not mediate the rapid effects of CRH on POMC gene expression. The Ca^{2+}-mediated induction of prolactin (Murdoch et al., 1985) and *c-fos* (Bravo et al., 1985; Fisch et al., 1987) gene expression, both of which are transcriptional regulated by PKC, are unaffected by PKC down-regulation (Bandyopadhyay & Bancroft, 1989), suggesting that PKC is not always an intermediate in the action of Ca^{2+} on gene expression.

Our studies indicate that intracellular Ca^{2+} is an important mediator of CRH-regulated POMC gene transcription in AtT20 cells. The additive transcriptional effects of combined CRH/8brcAMP and Bay K treatment on POMC primary transcript or hnRNA accumulation suggests cross-talk between Ca^{2+} and the well-characterized cAMP signaling systems. Such an interaction between signal transduction pathways has been demonstrated at the level of second messenger activation and protein kinase activity (for recent reviews see Blackshear et al., 1988; Hemmings et al., 1989). Recently, Greenberg and colleagues (1990) have demonstrated that independent Ca^{2+} and cAMP signaling pathways also converge on a common transcription factor and upstream regulatory element to activate *c-fos* transcription. Transcriptional responses to both cAMP and Ca^{2+} also appear to be

transduced by the same DNA element in the proenkephalin gene (Van Nguyen et al., 1990). We are currently identifying the cAMP and Ca^{2+} regulatory regions of the rat POMC promoter through deletion/mutation promoter analyses in order to further characterize the biochemical mechanisms mediating the regulatory link between these two major transmembrane signaling systems.

Acknowledgements

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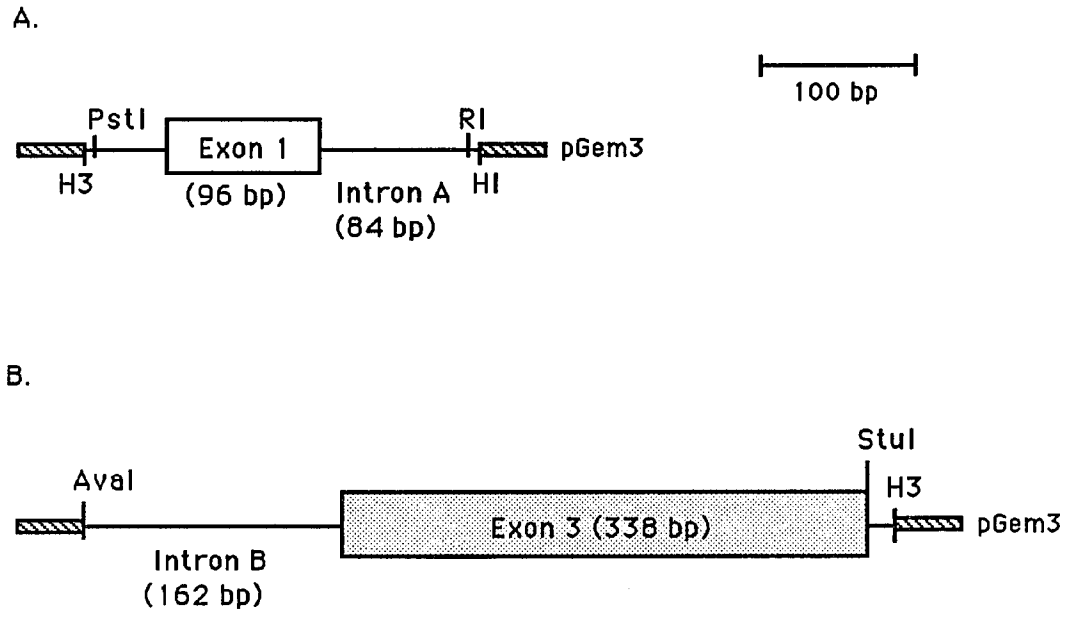


Figure 1. Schematic representation of the POMC genomic DNA vectors Mex1 (A) and Mex3 (B) used as templates for the generation of antisense and sense RNA probes in the SH/NP assays. The pMex 3 probe potects primary transcript, whereas the pMex1 probe protects both primary transcript and processing intermediate (designated as heteronuclear RNA or hnRNA).

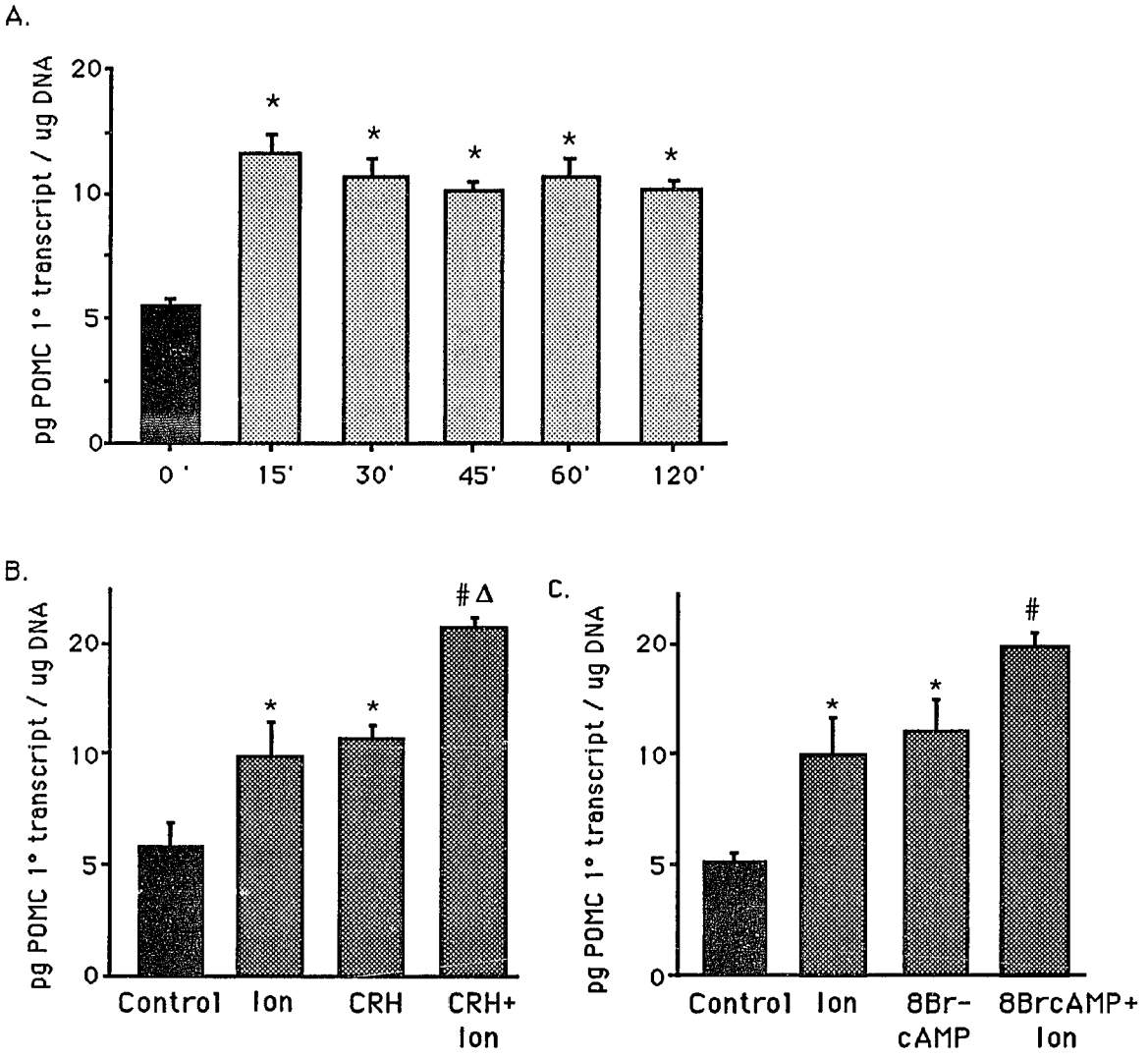


Fig. 2. Acute effect of the calcium ionophore ionomycin (Ion) on short-term changes in POMC gene expression in AtT20 cells. (A) Time-dependent effects of 1μM ionomycin on POMC primary transcript accumulation. (B) The effects of cotreating cells with 1μM ionomycin and 10nM CRH or (C) ionomycin and 1mM 8brcAMP (separate experiment) for 60 min on POMC primary transcript levels. Bars represent the mean of three independent determinations for each treatment ±SD. * $P > 0.05$ vs. vehicle-treated controls; #, $P > 0.001$ vs. vehicle-treated controls; Δ , $P > 0.001$ vs cells treated with ionomycin alone or CRH alone.

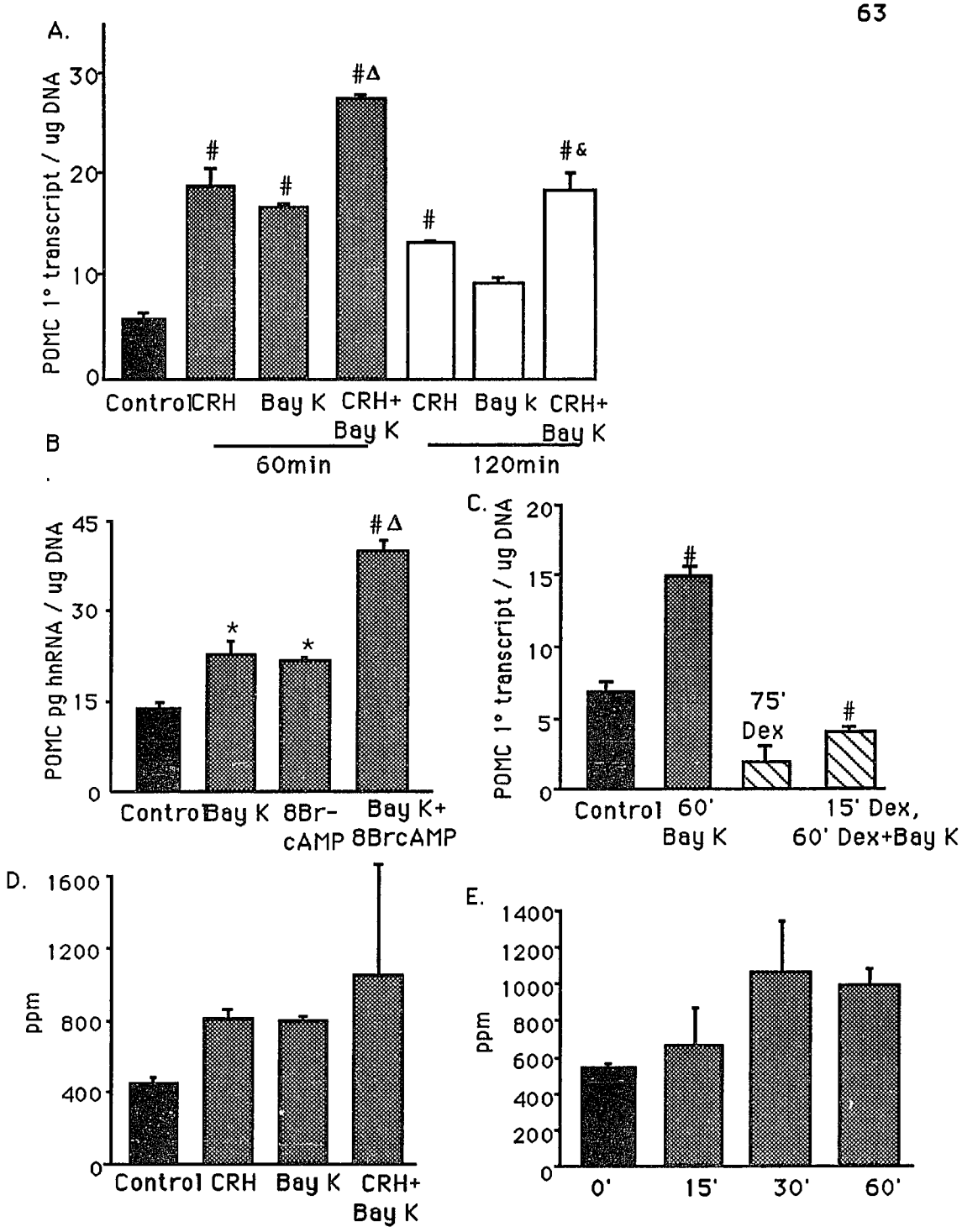


Fig. 3. The dihydropyridine agonist Bay K 8644 (Bay K) rapidly induces POMC expression in AtT20 cells. (A) The effects of 10nM CRH and/or 1μM Bay K for 60 min or 120 min or (B) 1mM 8brcAMP and /or 1μM Bay K for 60 min on POMC primary transcript accumulation relative to vehicle-treated controls. (C) Dexamethesone (Dex; 100nM) inhibits basal and Bay K (1μM)-stimulated POMC primary transcript accumulation. (D) POMC gene transcription was directly measured in a transcription run-on assay under the same treatment conditions as in (B) for 60 min or after 1μM Bay K treatment in a time-dependent fashion (E). Symbols represent individual points in ppm. #, P> 0.001 vs. vehicle-treated controls; Δ, P> 0.001 vs. cells treated with 60 min CRH or 60 min Bay K; &, P> 0.001 vs. cells treated with 120 min CRH or 120 min Bay K.

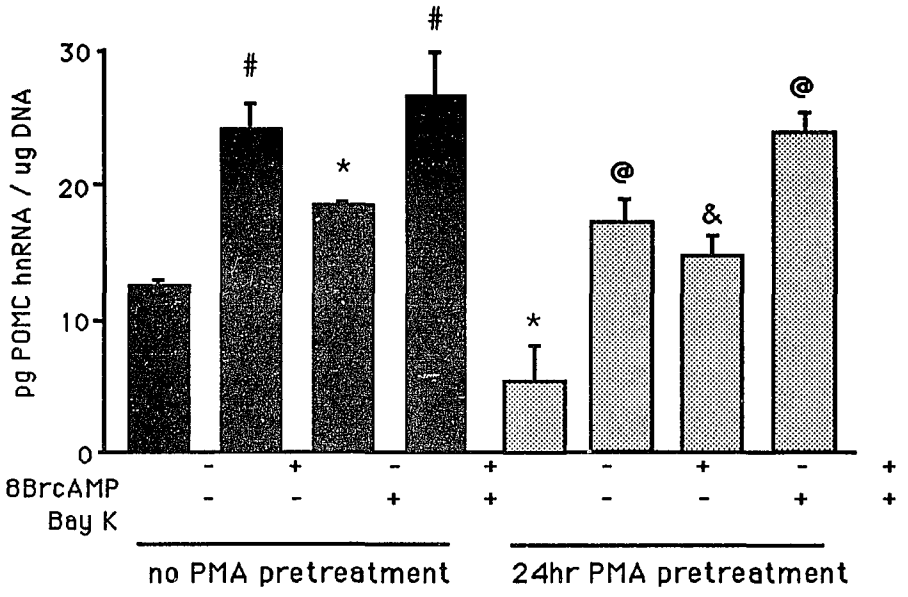


Fig. 4. The involvement of PKC in regulating short-term changes in POMC hnRNA levels in AtT20 cells. Cells were treated with 100nM PMA for 24 hrs prior to treatment with 1mM 8BrcAMP and/or 1μM Bay K for 60 min. Controls were given an equal concentration of DMSO (<1%) during the 24 hr incubation period. *, P > 0.05 vs. PMA-free controls; #, P > 0.001 vs. PMA-free controls; &, P > 0.05 vs. PMA-treated controls; @, P > 0.001 vs. PMA-treated controls.

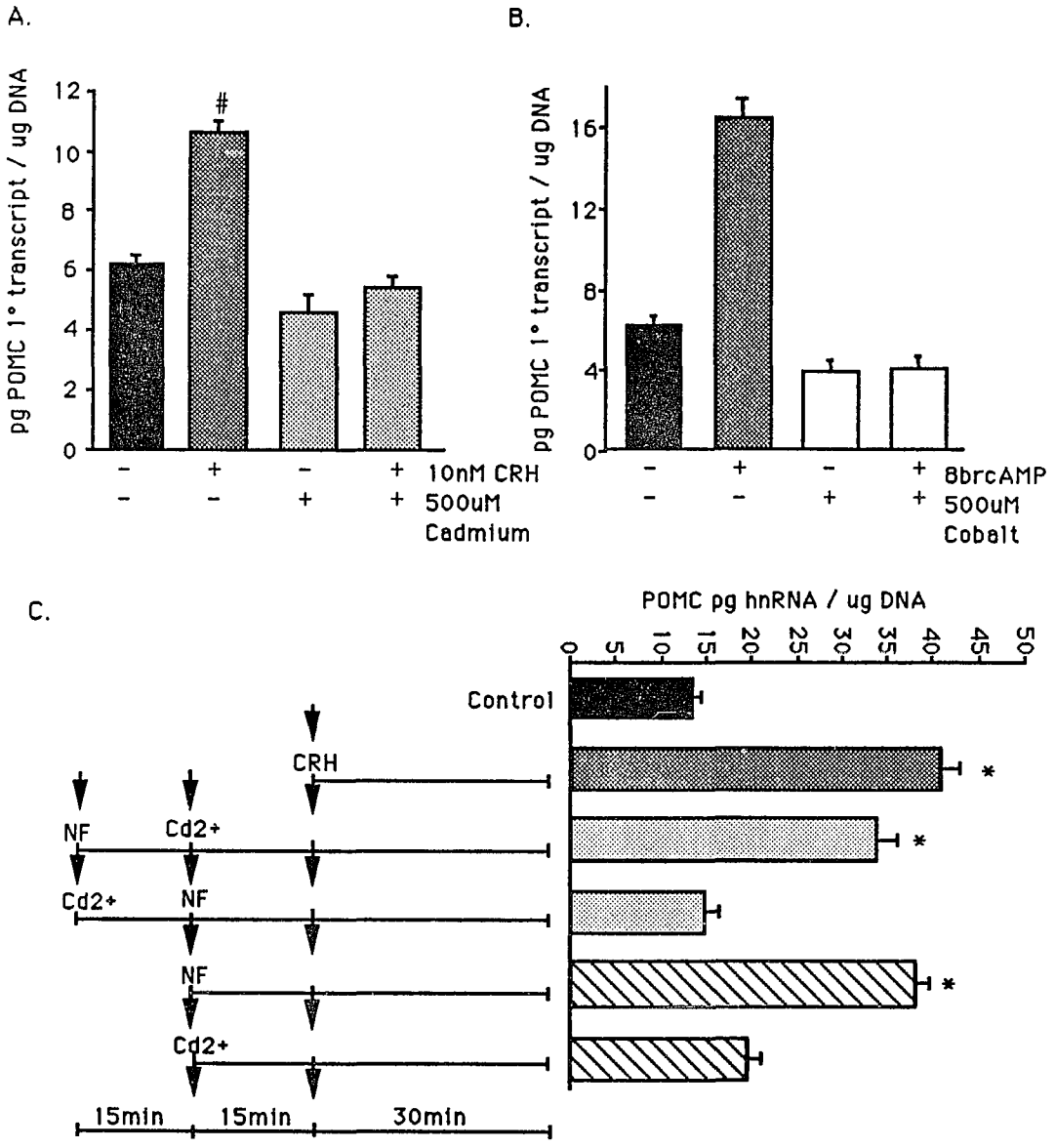


Figure 5. The pretreatment effects of cadmium (Cd2+), cobalt (Co2+) and nifedipine (NF) on 10nM CRH-induced nuclear POMC hnRNA accumulation in AtT20 cells. Pretreatment with 500μM Cd2+ (A) or 500μM Co2+ (B) for 15 min blocks the stimulatory effects of 60 min CRH treatment. (C) Cultures were treated with 50μM Cd2+ in the presence or absence of 1μM NF prior to 10nM CRH treament for 30 min as indicated. * P > 0.05 vs. vehicle-treated controls.

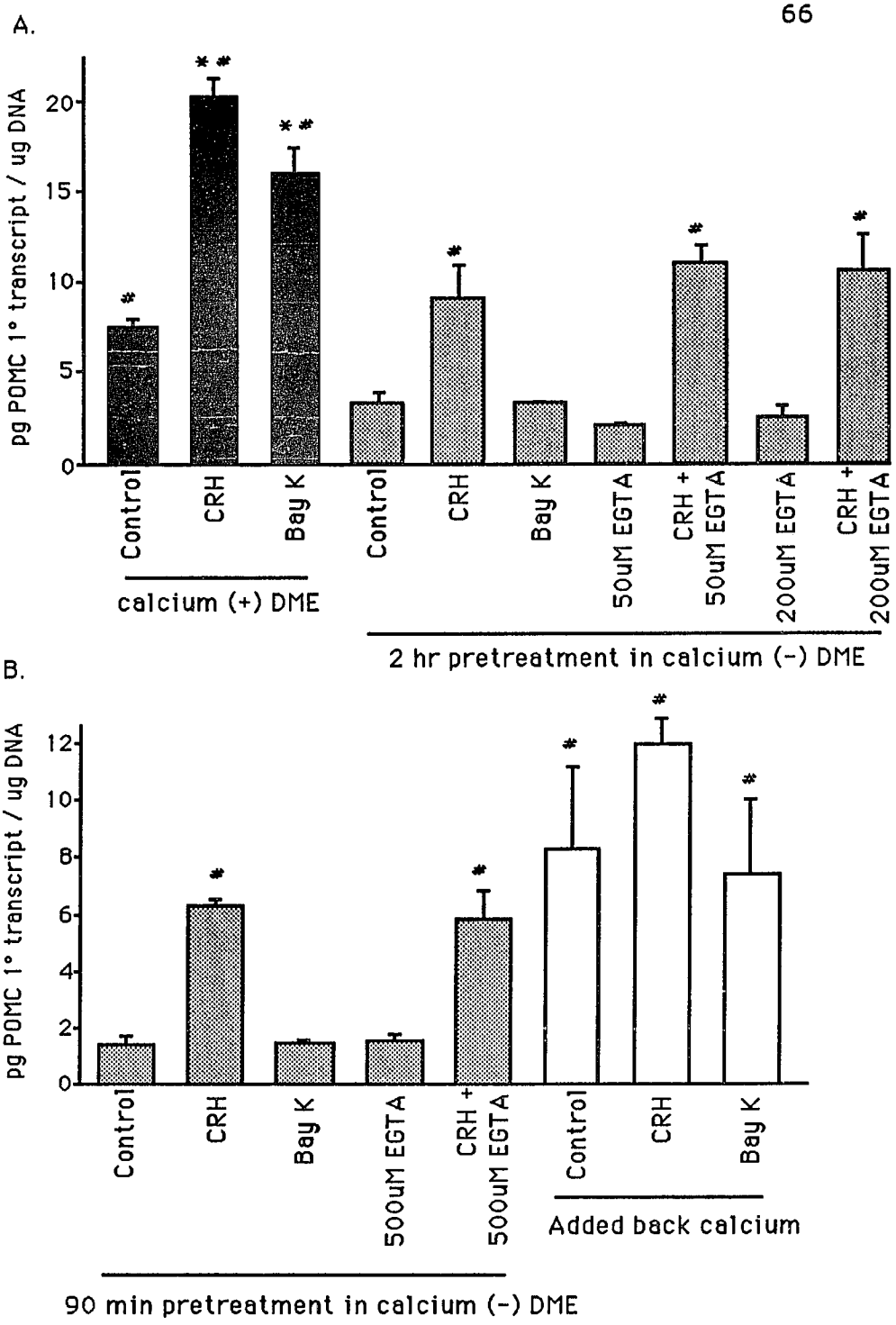


Figure 6. The effects of external calcium on POMC primary transcript accumulation in AtT20 cells. (A) Cells were treated for 60 min with 10nM CRH, 1μM Bay K or vehicle following either a 2 hr pretreatment with calcium-free DME in the presence of 0, 50, 200μM EGTA (stippled bars) or in calcium-supplemented DME (filled bars). (B) Following 90 min pretreatment in the calcium-free DME with or without added 500μM EGTA, the effects of 60 min 10nM CRH or 1μM Bay K on POMC primary transcript levels were measured in the continued absence of external calcium, with or without 500μM EGTA, (spotted bars) or after coaddition of calcium and 10nM CRH or 1μM Bay K to calcium-free DME (unfilled bars). *, $P > 0.001$ vs calcium treated controls. *, $P > 0.001$ vs calcium-free controls.

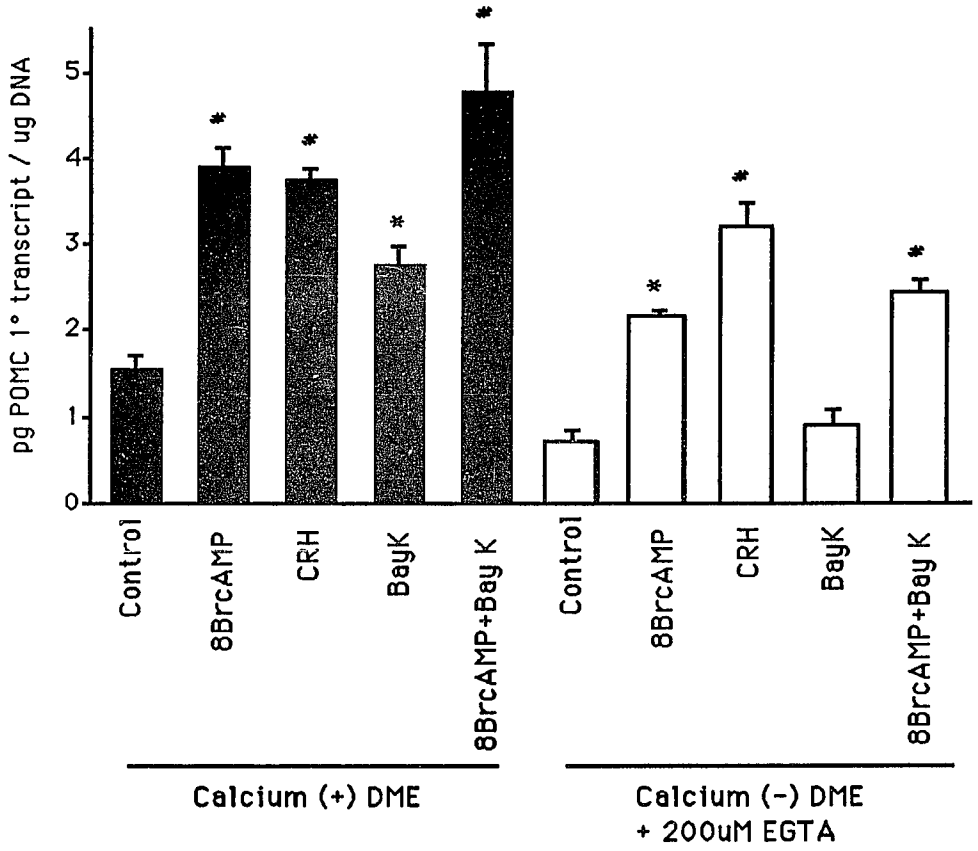


Fig. 7. The effects of 60 min treatment with 8brcAMP (1mM), CRH (10nM) and Bay K (1μM) on nuclear POMC primary transcript levels in the presence of calcium- supplemented DME (Calcium (+) DME; filled bars) or in the absence of external calcium with added 200uM EGTA (Calcium (-) DME; unfilled bars). * P > 0.05 vs. vehicle- treated controls; #, P > 0.001 vs calcium treated controls.

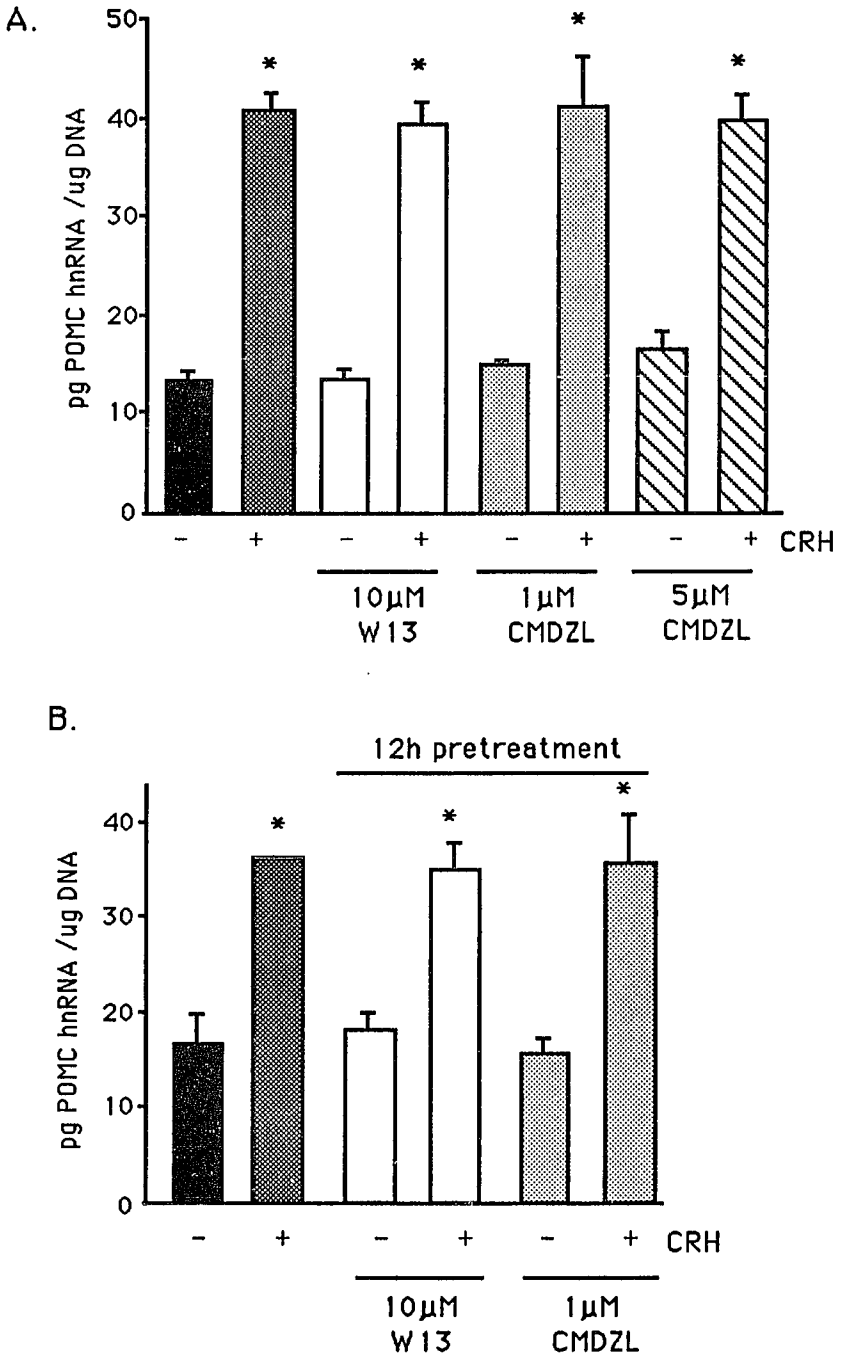


Figure 8. The effects of pretreating AtT20 cells for 1 hr (A) or 12 hrs (B) with the calmodulin antagonists CMDZL (spotted and striped bars) or W13 (unfilled bars) with or without subsequent 10nM CRH coincubation for 60 min on POMC hnRNA levels. * P > 0.001 vs. vehicle-treated controls.

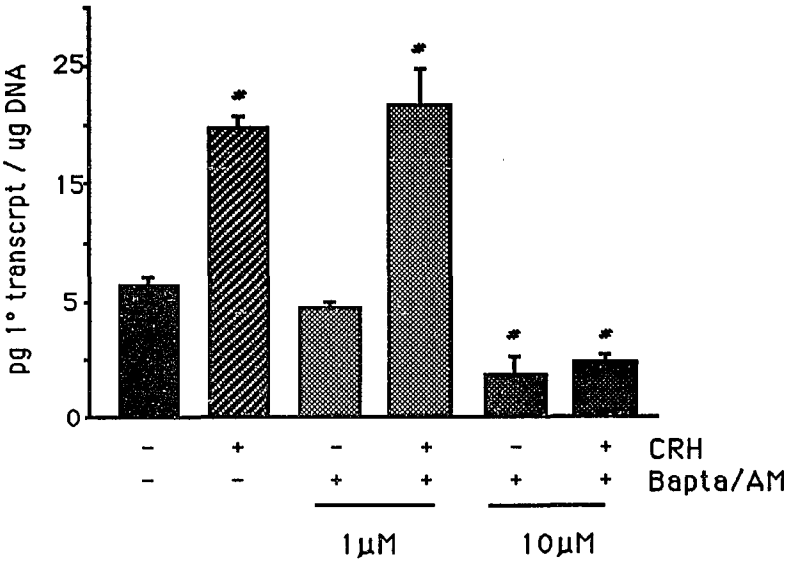


Figure 9. Cultures were treated with 1μM or 10μM Bapta/AM for 4 hrs in the presence of external calcium followed by costimulation with 10nM CRH for 60 min. Basal and CRH treated cultures free of Bapta/AM activity served as controls. *, P > 0.001 vs calcium treated controls.

VGCC antagonist	Control	VGCC antagonist	CRH	CRH+VGCC antagonist
Nifedipine (1,n) 1uM	18.1±4.4	19.2±4.0	27.9±0.9	27.1±3.3
Nitrendipine (1,n) 1uM	10.9±0.6	13.3±1.1	25.7±0.8#	24.9±0.9#
Verapamil (1,n) 10uM	15.0±2.5	12.1±0.9	25.2±1.2*	31.3±5.0#
Conotoxin (1,n) 10nM 100nM	2.3±0.5	2.5±0.6 2.4±0.8	7.0±0.4#	5.9±0.7# 6.1±0.3#

Table 1. Three different voltage-gated calcium channel (VGCC) antagonists, the dihydropyridine nifedipine and nitrendipine, the benzodiazepine verapamil and the natural snail toxin, conotoxin,were used to block the activity of different types of calcium channels in AtT20 cultures. Cultures were treated with a VGCC antagonist for 75 min, 10nM CRH for 60min, or cotreated with 10nM CRH and a particular VGCC anatagonist for 60 min following a 15 min pretreatment with the antagonist alone. Controls received ethanol (<1% final concentration) in experiments using the dihydropyridine antagonists and verapamil. All data is expressed as the mean of three independent determinations expressed as picograms POMC primary transcript and hnRNA relative to micrograms of DNA recovered ± SD. *, P > 0.05 vs. controls; #, P > 0.001 vs. controls. The calcium channel specificity for each antagonist is indicated in parantheses.

VGCC antagonist	Control	VGCC antagonist	CRH	CRH+VGCC antagonist
Nifedipine 1uM Exp. 1	649±98	501±108	1099±289@	955±20
Exp. 2	819±87	992±161	1687±597	1373±672
ω -conotoxin 100nM	64.5±7.8	83.7±21.4	126±5.0*	136.7±16.0*

Table 2. The effects of the VGCC antagonists nifedipine and conotoxin on the rate of POMC gene transcription measured in a transcription run-on assay in AtT20 cells. Cultures were treated with either 10nM CRH for 60min, VGCC antagonist for 75 min or pretreated for 15min with a VGCC antagonist followed by costimulation with CRH and a VGCC blocker. Data points represent the mean ± S.D. of three independent determinations for each treatment group and are expressed as parts per million. @, P> 0.05 vs. cells treated with nifedipine alone; *, P>0.05 vs. controls.

Chapter 4

Multiple Intracellular Mediators Regulate POMC Promoter CAT Activity in Transiently Transfected AtT20 Cells.

Abstract

Proopiomelanocortin (POMC) gene transcription in the anterior pituitary corticotrope is stimulated by hypothalamic releasing factors via intracellular mediators and repressed by glucocorticoids. To delineate sequences important for corticotropin releasing hormone (CRH) and Ca^{2+} upregulation of transcription of the POMC gene, a fragment of the rat POMC genomic DNA containing 706 bp of the 5' flanking sequence, including the POMC promoter, transcription initiation site and 63 bp of exon 1, was fused to the gene encoding the bacterial chloramphenicol acetyl transferase (CAT) gene (p706XP-CAT) and transiently transfected into AtT20 cells. After 16-24 hrs exposure to secretagogues, cells were harvested and assayed for their ability to acetylate [^{14}C]-chloramphenicol. CRH (10nM) or 8BrcAMP (1mM) elevated CAT activity 5.3-fold and 12.5-fold respectively relative to vehicle-treated controls. Cotreating cells with 100nM dexamethasone (Dex) and CRH or 8BrcAMP attenuated CRH induction to 2.1-fold and 8BrcAMP to 5.2-fold, whereas Dex alone had no significant effect. Cell treatment with the dihydropyridine agonist Bay K 8644 (Bay K,1μM) had no effect on p706XP-CAT activity by itself, however it potentiated 10nM CRH stimulation 4.7-fold over treatment with CRH alone. Both the divalent metal ion cadmium (50 or 500uM) and the cytosolic Ca^{2+} chelator, Bapta/AM (10μM) decreased basal and blocked CRH-inducible CAT activity in p706XP-CAT transfected cells. To further delineate sequences important for cAMP- and Ca^{2+}-mediated upregulation of POMC gene transcription, a series of POMC 5' flanking deletions were ligated into a CAT cassette: -430 to +63 bp (p430BP-CAT), -320 to +63 bp (p320SP-CAT) and -234 to +63 bp (p234P-CAT). In p430BP-CAT transfected cells, CRH induced CAT activity 6.4-fold, Bay K 1.1-fold and cotreatment with CRH and Bay K 14.6-fold over controls. Similar fold induction was obtained with p320SP-CAT tranfected cells, suggesting promoter elements upstream of -320 bp are not critical for positive regulation. Unlike the larger 5' deletion contructs, p234P-CAT transfected cells treated with Bay K resulted in a 2-fold

elevation in CAT activity over controls. A smaller POMC promoter fragment from -236 to -133bp containing sequences homologous to an AP-2 regulatory element, fused to a thymidine kinase promoter driven CAT cassette (p236/133-tkCAT), strongly conferred CRH/Ca^{2+} inducibility. CRH (10nM) induced CAT activity 14-fold over controls in p236/133-CAT transfected cells. Interestingly, 1μM Bay K elevated CAT activity 7.8-fold in p236/133-CAT transfected cells, and cotreatment with 10nM CRH and Bay K resulted in an enhanced 16-fold induction over vehicle-treated controls. Together, these studies indicate that cytosolic elevations of either cAMP or Ca^{2+} stimulate POMC gene transcription to a lesser extent than does the combination of cAMP and Ca^{2+} through a discrete DNA element located in the POMC promoter.

Introduction

Proopiomelanocortin (POMC) gene expression in anterior pituitary corticotropes is positively regulated by the hypothalamic peptides corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), and negatively regulated by the adrenal corticosteroids (see review, Lundblad & Roberts, 1988). The interaction between these stimulatory and inhibitory agents at the level of transcription and processing of heteronuclear RNA (see chapters 2 and 3) indicates that specific regions within the POMC 5' flanking sequence is regulated by hormones via multiple second messenger pathways. A number of reports have demonstrated that most, if not all, forms of transcriptional regulation by hormones are mediated by specific nucleotide sequences usually present within the 5' flanking region of genes. A number of DNA control elements have been characterized as the binding sites for specific nuclear proteins in response to the activation of discrete second messenger pathways, such as the cAMP response element (CRE) (Montminy et al., 1990), the phorbol ester-inducble element (Hoeffler et al., 1989), and the recently identified Ca^{2+} responsive element (Sheng et al., 1988; Van Nguyen et al., 1990). The aim of this study is to characterize the cAMP and Ca^{2+} regulatory regions within the POMC gene 5' flanking region through the use of fusion gene constructs in order to better understand the mechanisms by which CRH exerts its stimulatory effect on POMC gene expression.

Transcriptional responses to intracellular cAMP have been shown to be mediated through discrete regulatory sequence elements identified in a variety of neuropeptide genes including somatostatin, VIP and proenkephalin (see review, Goodman, 1990). These genes share similar CREs, some of which contain one or more copies of the conserved palindromic sequence motif 5' TGACGTCA 3'. The transcriptional response to cAMP involves the binding of transacting factors to the CRE. The POMC gene, however, does not contain a consensus CRE in its promoter region. Through the use of POMC promoter fusion gene constructs in a transient CAT expression system, Roberts and colleagues (1987) have previously identified two CRH/cAMP responsive regions at -478 to -320 bp and -236 to -133 bp relative to the transcription start site. Other neuropeptide genes known to be responsive to cAMP (eg., growth hormone and prolactin) also do not contain the palindromic CRE in their 5' flanking sequence, strongly suggesting that cAMP regulates neuropeptide gene expression by multiple transcriptional mechanisms. In fact, both *in vitro* dimethyl sulfate methylation protection and DNase protection assays and *in vitro* gel retardation assays with the POMC gene promoter have revealed a complex pattern of CRH-

regulated protein binding domains, however, the identities of the proteins involved remain to be determined (Lundblad et al., manuscript in preparation).

Ca^{2+} responsive elements have been recently characterized in the 5' upstream region of a number of genes, including *c-fos* and prolactin (Gilman, M.Z., 1988, Sheng, M., et al, 1988; Jackson & Bancroft, 1988). The mechanism by which a cytosolic Ca^{2+} signal is transduced into the nucleus to activate gene transcription has been well characterized for the *c-fos* proto-oncogene. Through promoter deletion analyses, Greenberg and colleagues have identified a Ca^{2+} responsive element (CaRE) which is regulated by membrane depolarization and Ca^{2+} influx, approximately 60 bp upstream of the mRNA start site of the human *c-fos* gene (Sheng et al., 1990). This CaRE, previously shown to confer *c-fos* transcriptional regulation by cAMP (Sassone-Corsi et al., 1990; Fisch et al., 1989), also contains a sequence (-TGACGTTT-) that resembles the consensus 8 bp palindromic CRE (-TGACGTCA-). CaRE oligonucleotides confer inducibility by cAMP, and fine mutagenesis of the CaRE does not dissociate Ca^{2+} and cAMP regulation, suggesting that both second messenger pathways use a common, rather than a distinct, target protein(s) that interacts with the CaRE. A precedent for this is seen where both cAMP and Ca^{2+} signaling mechanisms converge at the level of nuclear factor cAMP regulatory element binding protein (CREB) activation (Sheng et al., 1990).

Although the molecular mechanisms by which cAMP regulates neuropeptide gene expression have been well-characterized (Comb et al., 1986; Montminy et al., 1986; Hyman et al., 1988), relatively little is known about the mechanisms by which Ca^{2+} produces transcriptional effects. In this paper, we show that Ca^{2+}-induced activation of POMC gene expression by Bay K-induced calcium entry into AtT20 cells is mediated through a Ca^{2+}-responsive DNA element within -236 and -133 bp (relative to the transcription start site) of the POMC 5' flanking sequence using the AtT20 cell line as a substrate for gene transfer experiments. This region of the POMC promoter also contains a CRH-responsive element, suggesting that regulation of gene expression by Ca^{2+} and cAMP may converge on a single DNA element.

Methods

Cell culture techniques. Mouse AtT-20 D16/16 tumor cells were cultured in Dulbecco's Modified Eagle medium (DME, Gibco) supplemented with 10% horse serum and 5% fetal calf serum. Cells were plated in 35-mm diameter culture dishes (Corning) at an initial density of 1.5 x 10^5 cells/dish and were grown in a 10% CO2 humidified incubator at 37°C. Cells were tranfected 3-4 days after subculturing (60-80% confluency). Cells were passaged at weekly intervals, and stock cultures were rethawed after 10 passages.

Plasmid constructions. A 10 kb EcoRI fragment of DNA containing the rat genomic POMC gene was isolated from a Sprague-Dawley rat genomic library (Eberwine & Roberts, 1984). A fragment of the 5' flanking region of the POMC gene from this 10 kb sequence was isolated and ligated to pCAT or ptkCAT to generate p704XP-CAT (Fig. 1A) and p704XP-tkCAT (Fig. 1B) fusion constructs respectively. The p704XP-CAT vector contains -704 to +63 bp of the POMC promoter, including the TATA box, the transcription initiation site, and a portion of exon 1, fused to the bacterial reporter gene chloramphenicol acetyltransferase (CAT) coding sequence harbored in a pUC19-derived plasmid. The p704XP-tkCAT plasmid contains -704 to -37 bp of the POMC 5' flanking sequence linked to a nonregulated, constituitively activated promoter, the Herpes Simplex Virus thymidine kinase (tk) promoter driving the CAT cassette (for details see Lundblad et al., manuscript in preparation). These POMC promoter fusion CAT constructs also contain SV40 splicing and polyadenylation signals derived from pSV2CAT (Gorman, 1985).

A nested series of 5' deletions of the POMC promoter sequences were generated using existing restriction sites in the parent p704XP-CAT fusion vector (for details see Roberts et al., 1987). A fragment of the POMC promoter extending from -236 to + 63 bp was generated by Bal31 digestion followed by blunt end repair with the Klenow fragment of DNA polymerase I. Internal deletions were similarly generated, digesting the parent p706XP-CAT vector with pairs of restriction enzymes and repairing the ends with DNA polymerase. A promoterless CAT construct (p0-CAT) serves as a negative control for regulated CAT activity, and the reference plasmid pRSV-bGAL contains the RSV promoter linked to the coding sequence of b-galactosidase, harbored in a pUC derivative plasmid.

Transfection and Enzyme Assays. AtT20 cells were washed once with serum-free media and returned to the incubator for 2 hrs. Cells were then transfected with lipopolyamine-coated supercoiled plasmid DNA for 10-12 hrs (Behr, J-P et al., 1989). At the end of the transfection period, the transfectant was removed and cells were allowed to recover for > 12 hrs in serum-containing medium. Cultures were treated with hormones or pharmacological agents for 16-24 hrs starting approximately 24 hrs posttransfection in the presence of serum-free medium. After treatments, cells were washed with and harvested in 1ml of cold 1x phosphate buffered saline, pH 7.4 (1xPBS), centrifuged and resuspended in 100ul of 0.25M Tris-Cl, pH 7.8. Whole cell extracts were prepared as described by Gorman et al. (1982). One-half of the lysate (representing about ug of protein) from each transfection was assayed for CAT activity by thin-layer chromatography. Lysates were incubated with 1.0μCi (1 Ci=37 GBq) of [^{14}C]-chloramphenicol and 0.2mM acetyl-Coenzyme A in 150mM Tris-Cl, pH 7.8 for 3 hrs at 37°C. Under these conditions, CAT activity was linear with time. Transfection efficiencies were normalized using b-galactosidase activities derived from cotransfected pRSV-bGAL. Calculation of percent conversion for CAT activity was carried out as previously described (Roberts et al., 1987).

Statistical analysis. Each experimental condition was repeated in triplicate, corrected for transfection efficiency and expressed as a mean value $\pm$ standard deviation. The data was evaluated by a one factor ANOVA and differences with $P < 0.05$ (Fisher PLSD), as indicated, were considered significant.

Results

Previous gene transfer studies from this laboratory and others demonstrate that the 5' flanking region of the POMC gene including 706 bp upstream from the transcription start site is sufficient for CRH and cAMP. This POMC promoter region has been shown important for repression by glucocorticoids (Drouin et al., 1989; Charron & Dourin, 1986) and contains the information necessary for pituitary-specific regulation of POMC gene transcription (Jeanotte et al., 1987; Tremblay et al., 1988). In this study, a fragment of rat POMC genomic DNA containing 706 base pairs of the 5' flanking sequence was fused to a promoterless CAT gene (designated p706XP-CAT, Fig. 1A) and transiently introduced into AtT20 cells by lipofection (Behr, J-P et al., 1989). RNA isolated from cells stablely transfected with this CAT fusion gene shows transcription initiation from the correct site (data not shown), indicating that this construct contains all the necessary functional elements of the POMC promoter. The constituitive expression of p706XP-CAT was about 5% of the level of pRSV-CAT, a plasmid that contains the Rous sarcoma virus promoter fused to the CAT structural gene.

Figure 1C shows the regulation of POMC promoter directed CAT activity in p706XP-CAT-transfected AtT20 cells treated with either CRH (10nM), 8brcAMP (1mM), Dex (100nM) and Bay K (1μM) for approximately 18 hrs. CRH and 8brcAMP increase CAT activity 5- or 12-fold respectively relative to untreated controls. Treatment of cells with the synthetic glucocorticoid dexamethasone (Dex) shows little inhibitory effect as a result of the low basal CAT activity. However, cotreating cells with Dex and either CRH or 8brcAMP attenuates CAT induction to 2.5- or 4-fold respectively relative to controls, a level of expression significantly less than treatment with CRH or 8brcAMP alone. Although Bay K treatment alone has been demonstrated to acutely increase POMC gene transcription (see chapter 3), it had no significant effect on transcription of p706XP-CAT. All the pharmacological agents and hormones in this paper had no significant effect on transcription of a promoterless CAT fusion gene (pO-CAT), which was barely above background CAT activity (data not shown).

Although mobilizing cytosolic Ca^{2+} with Bay K had no effect on p706XP-CAT activity (Fig. 1B), 50 and 500μM Cd^{2+} treatment decreased basal and blocked CRH-stimulated CAT activity (Fig. 2A). Further evidence supporting the regulatory effect of Ca^{2+} on POMC gene transcription was obtained by treating p706XP-CAT transfected cells with a membrane permeable intracellular Ca^{2+} chelator, Bapta/AM. Cotreating cells with 10nM

CRH and 10μM Bapta/AM for 18 hrs decreased basal and blocked CRH-induced CAT activity (Fig. 2B).

5' deletion constructs. To delineate sequences important for CRH and Bay K regulation of POMC gene transcription, progressive 5' deletions of the 5' flanking sequences of p706XP-CAT were linked to the CAT reporter gene. These CAT expression vectors were tested for basal unstimulated activity and CRH and/or Bay K induction in AtT20 cells. In cells transfected with p430BP-CAT or p320SP-CAT, 10nM CRH treatment induced CAT expression approximately 7- or 5-fold respectively over controls. Bay K (1μM) treatment alone had no significant effect on POMC CAT activity in either p430BP-CAT or p320SP-CAT transfected cells. However, the combination of 10nM CRH and 1mM Bay K enhanced CAT activity greater than treatment with either agent alone, 14-fold in p430BP-CAT transfected cells and 12-fold in p320SP-CAT transfected cells relative to p706XP-CAT untreated controls. Thus, full cAMP and/or Ca^{2+} responsiveness is obtained in the absence of sequences upstream of -320 bp of the POMC promoter. Basal CAT activity obtained with these various 5' deletion POMC promoter CAT constructs were comparable to that obtained with the p706XP-CAT parent vector.

Treating p234P-CAT transfected cells with 10nM CRH increases CAT activity 12-fold over controls (Fig.3B), a greater level of CRH induction than that obtained with the previously described 5' deletion constructs. Although 1μM Bay K treatment has no stimulatory effect on p706XP-CAT, p430BP-cat or p320SP-CAT transfected cells, Bay K induced CAT expression 2- to 3-fold in cells transfected with a smaller fragment of the POMC promoter (-234 to +63 bp) although this effect was not significant in this experiment. Combined CRH and Bay K treatment resulted in a 17-fold induction relative to controls, greater than treatment with either agent alone. Taken together, these data suggest a model in which cAMP plays a permissive role for the actions of Ca^{2+} on POMC gene transcription.

CRH-regulated promoter regions. Portions of the POMC promoter were linked 5' to the HSV tk promoter driven CAT cassette as depicted in Figure 1B. Previous transfection studies from our laboratory (Roberts et al., 1987) have used these heterologous promoter constructs and identified two regions of the POMC promoter containing sequence information sufficient for CRH and forskolin regulation, located at -236 and -133 bp 5' to the start of transcription, and a weaker more distal element bewteen -320 and -477 bp. In this paper, we assayed the ability of the heterologous promoter construct containing -236 to -133 bp of the 5' POMC flanking sequence (p236/133-tkCAT) to confer CRH- and Bay K-

inducibility in AtT20 cells. CRH (10nM) induced CAT activity 8-fold over controls in p236/133-tkCAT transfected cells. Treatment with 1μM Bay K by itself upregulated p236/133-tkCAT activity 7-fold and cotreatment with Bay K and CRH resulted in a greater 15-fold induction relative to vehicle-treated controls. 8brcAMP (1mM) treatment, in the presence or absence of Bay K, stimulated p236/133-tkCAT activity with the same level of induction as that obtained with CRH (data not shown). These results indicate that sequences between -236 and -133 bp are sufficient for conferring cAMP and Ca^{2+} inducibilty upon a heterologous promoter. The level of induced CAT activity produced by CRH and Bay K in p236/133-tkCAT transfected cells is much higher than that obtained with the full length POMC promoter fragment (p706X-tkCAT), indicating the presence of inhibitory DNA regulatory elements outside of this putative Ca^{2+}/cAMP regulatory region.

To further examine whether the POMC promoter region between -236 and -133 bp contains the minimum sequence information sufficient for cAMP and/or Ca^{2+} regulation of expression of POMC fusion genes in AtT20 cells, we transfected cells with a heterologous promoter CAT construct missing the region from -320 to -37 bp of the POMC 5' flanking sequence (Δ320/37-tkCAT) (Fig. 4). Although treatment with 10nM CRH induced CAT activity 4-fold over controls, 1μM Bay K alone had no effect. Combined CRH and Bay K treatment did not result in an additive induction over treatment with CRH alone despite the fact that additive inducibility was obtained with the full length POMC promoter fragment (p706X-tkCAT) and the smaller cAMP/Ca^{2+} regulated fragment (p236/133-tkCAT) (Fig. 4).

Somatostatin CRE. We next examined the ability of a fragment of the somatostatin 5' flanking sequence containing a well-characterized cAMP regulatory region, to confer cAMP and/or Ca^{2+} inducibility in AtT20 cells (Fig. 5). Cells were transiently transfected with a 31 bp portion of the somatostatin promoter containing the 10 bp palindromic CRE fused to the CAT gene (ssΔ-71), a generous gift from R. H. Goodman. CRH (10nM) induced CAT expression 4-fold over controls. Bay K (1μM) treatment alone had no effect alone but potentiated induction by CRH relative to controls. In the same experiment, p236/133-tkCAT activity was measured under identical treatment conditions as described for ssΔ-71 transfected cells. Both CRH and Bay K treatment induced CAT activity over controls in p236/133-tkCAT transfected cells with levels of induction similar to that previously described in Figure 4. The effects of combined Bay K and CRH treatment were significantly greater than treatment with CRH or Bay K separately on this promoter fragment.

Discussion

The experiments presented in this paper demonstrate that the stimulatory effects of CRH and Bay K are mediated through a specific region of the 5' flanking sequence of the POMC gene. In contrast to the CRH stimulatory effects, Bay K treatment alone failed to regulate CAT activity in cells transfected with the fragment of the POMC promoter from -704 to +63 bp (relative to the transcription start site), even though previous studies have demonstrated that acute treatment with Bay K stimulates the rate of POMC gene transcription and the induces POMC primary transcript levels (Chapter 3). The effects of cadmium or Bapta/AM on this POMC promoter/reporter construct, however, paralleled previous results measuring POMC hnRNA levels (Chapter 3). In addition, the stimulatory effects of Bay K on POMC promoter CAT activity became apparent when combined with CRH treatment, suggesting an interaction between cAMP and Ca^{2+} signaling pathways for maximal activation of POMC gene transcription. Therefore, in light of these results, we were certain we could identify a possible Ca^{2+} regulatory element(s) within the POMC gene.

Bay K significantly enhances the stimulatory effects of CRH in all 5' deletion promoter constructs tested, indicating that the POMC 5' flanking sequence downstream of -236 bp, relative to the transcription start site, is sufficient for conferring cAMP and Ca^{2+} inducibility. While treatment with Bay K alone had no effect in the 5' extended constructs, slight activation of the -236 construct was observed, suggesting that regions 5' to -236 are suppressing POMC promoter activation by Ca^{2+} in this configuration. While it is possible that the rat POMC gene promoter behaves differently from the endogenous mouse POMC gene, which is clearly activated by Bay K alone, preliminary results from our laboratory (Levin et al., manuscript in preparation) indicate that the endogenous rat POMC gene in anterior pituitary primary culture is regulated by Ca^{2+} similarly to AtT20 cells. It is likely that other cis-acting elements within the POMC promoter, distinct from this cAMP/Ca^{2+} regulatory region, may suppress Bay K-stimulated POMC promoter activity.

5' deletion of the POMC promoter to -133 bp (relative to the transcription start site) dramatically reduces basal CAT activity (>90%) and eliminates induction by CRH/cAMP (Lundblad et al., manuscript in preparation). Therefore, one could conclude that cAMP and Ca^{2+} regulatory elements are colocalized within -234 to -133 bp of the POMC 5' flanking sequence. Analysis of this region in a heterologous construct demonstrates that it in fact confers inducibility to CRH or Bay K alone. Furthermore, combined CRH and Bay

K effects on this -236 to -133 bp POMC promoter fragment were additive, resulting in a level of induction similar to combined treatment effects on the full promoter fragment (-704 to -37). Ca^{2+}-inducibility of this promoter region further supports the idea that regions flanking this fragment (-236 to -133 bp) somehow suppress Ca^{2+} regulated activity of the promoter/reporter construct.

To determine whether this cAMP/Ca^{2+} regulatory region is in fact necessary for conferring CRH and/ Bay K inducibility on a heterologous promoter, we deleted -320 to -37 bp from the parent p706XP-tkCAT fusion construct and tested its ability to be regulated by CRH and/or Bay K. CRH continued to induce transcription, consistent with the presence of multiple cAMP regulatory elements within the POMC 5' flanking sequence (Roberts et al., 1987). However, Bay K could not confer induction on this heterologous promoter deletion construct, either alone or in combination with CRH, further supporting the presence of a functional Ca^{2+} regulatory element(s) within the first 320 bp sequences of the POMC promoter.

In this paper, we have identified the POMC DNA regulatory regions important in second messenger regulated POMC gene transcription. In addition, our results demonstrate that while the elevation of Ca^{2+} is sufficient for activation of the gene in a particular configuration, the cAMP signal transduction pathway cooperates positively in producing an intensified biological signal. Elevation of intracellular cAMP by CRH or 8brcAMP treatment in the presence of a Ca^{2+} channel blocker Cd^{2+}, however, had no effect on POMC promoter activity. In addition, chelating intracellular Ca^{2+} by Bapta/AM blocked CRH inducibilty even though cAMP was elevated. Thus, elevation of cAMP in the absence of a rise in intracellular Ca^{2+} is not sufficient for activating POMC gene transcription.

This interaction between cAMP and Ca^{2+} signaling pathways may occur at the level of binding of proteins to DNA, interaction of non-DNA binding domains of proteins or recruitment of additional proteins to the transcriptional complex. Cross-talk between the two signal transduction pathways may also result from enhanced adenylate cyclase or protein kinase activity, although the former is unlikely since there is no precedent for this interaction in the literature. More importantly, the diversity of second messenger systems present in corticotropes indicates that multiple mechanisms must exist for regulating POMC gene expression in response to complex incoming signals.

Since results from our laboratory indicate that the POMC gene responds to multiple regulatory cues through several cis-elements scattered over large stretches of DNA, it is likely that the promoter environment plays an especially important role in the regulation of the gene. Linking fragments of the POMC 5' flanking sequence to a heterologous promoter, then testing this chimeric promoter for transcriptional activity following transfection into cells cannot always correctly identify the precise functional role that second messenger responsive elements exert on their native intact promoter (see review by Roesler et al., 1990). In fact, cooperative interactions between the cAMP and Ca^{2+} signal transduction pathways are likely to be mediated between functionally and physically distinct elements within the promoter. In light of the complexity of the POMC promoter environment, we are currently using a series of linker-scanning mutants to further characterize the functional elements within the POMC promoter regulated by the cAMP and Ca^{2+} intracellular mediators.

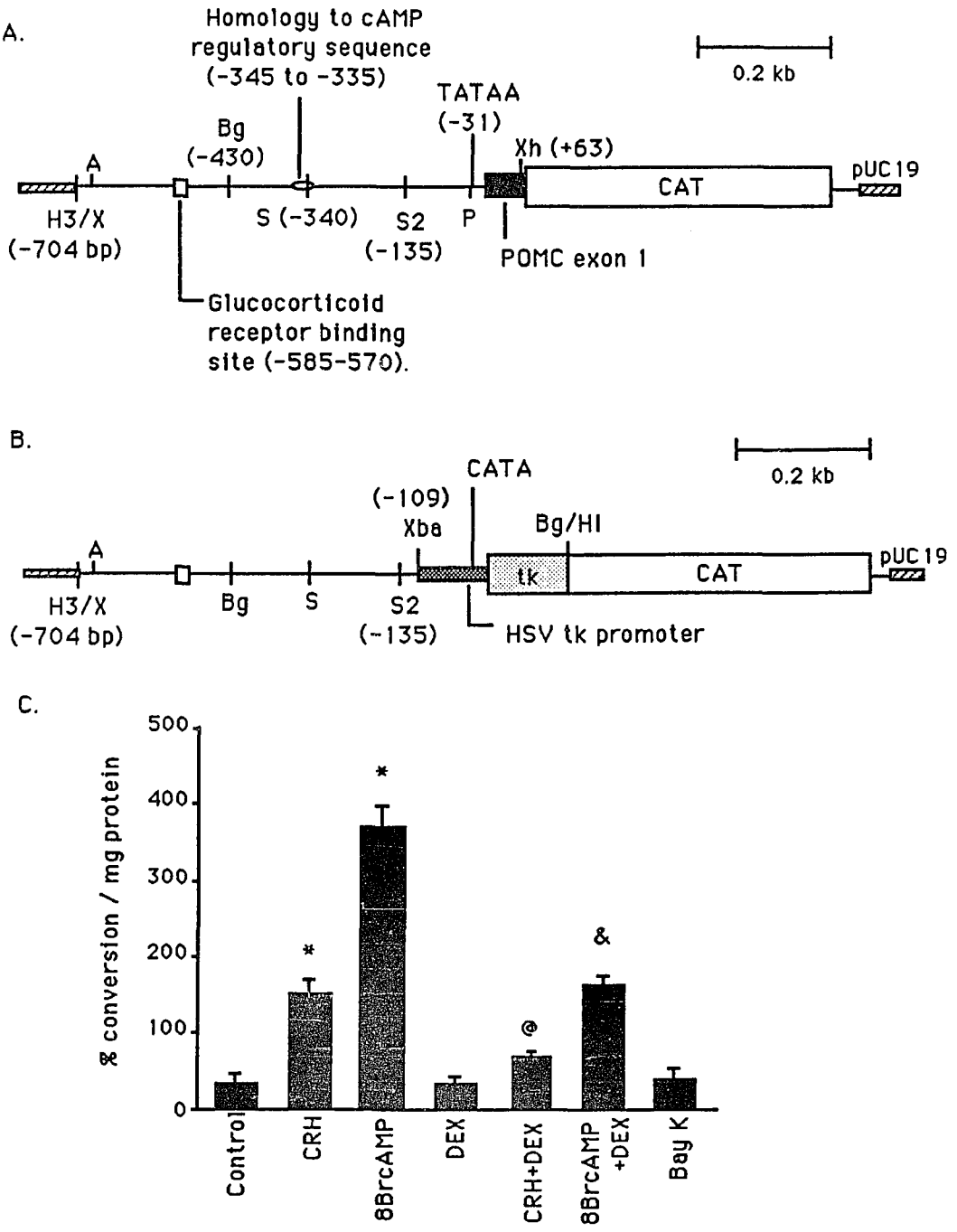


Figure 1. Schematic representation of the full length POMC promoter CAT fusion constructs p704XP-CAT (A) and p704X-tkCAT (B). (C) AtT20 cells were transiently transfected with a rat POMC promoter chloramphenicol acetyl transferase (CAT) fusion construct containing -706 to +63 bp of the POMC 5' flanking sequence relative to the mRNA transcription site (p706P-CAT). Cells were treated for 16 hrs with agents that either positively (10nM CRH, 1mM 8brcAMP and 1μM Bay K) or negatively (100nM Dex) regulate POMC gene expression. Data represents the mean of three independent determinations for each treatment ±S.D.. *, P> 0.05 vs. controls; @, P> 0.05 vs. CRH-treated cells; &, P> 0.05 vs. 8brcAMP-treated cells.

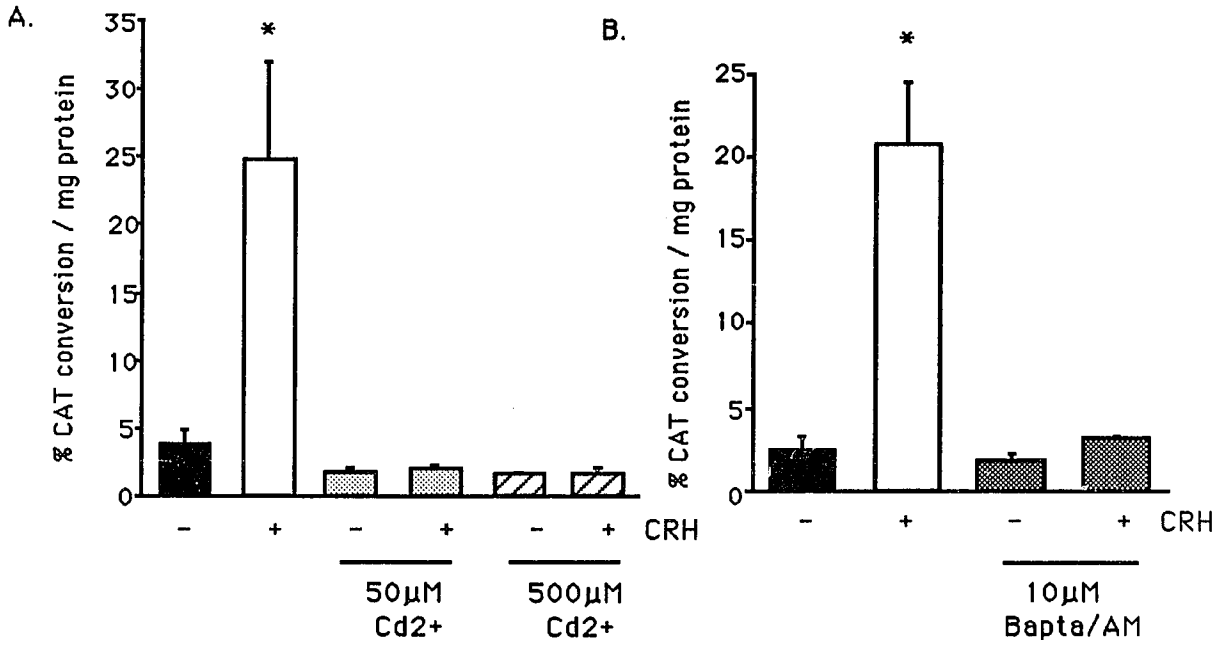


Figure 2. The inhibitory effects of 50 or 500µM cadmium (A) or 10µM Bapta/AM (B) on basal or 10nM CRH-cotreated p706P-CAT-transfected cells. *, P>0.05 vs. controls.

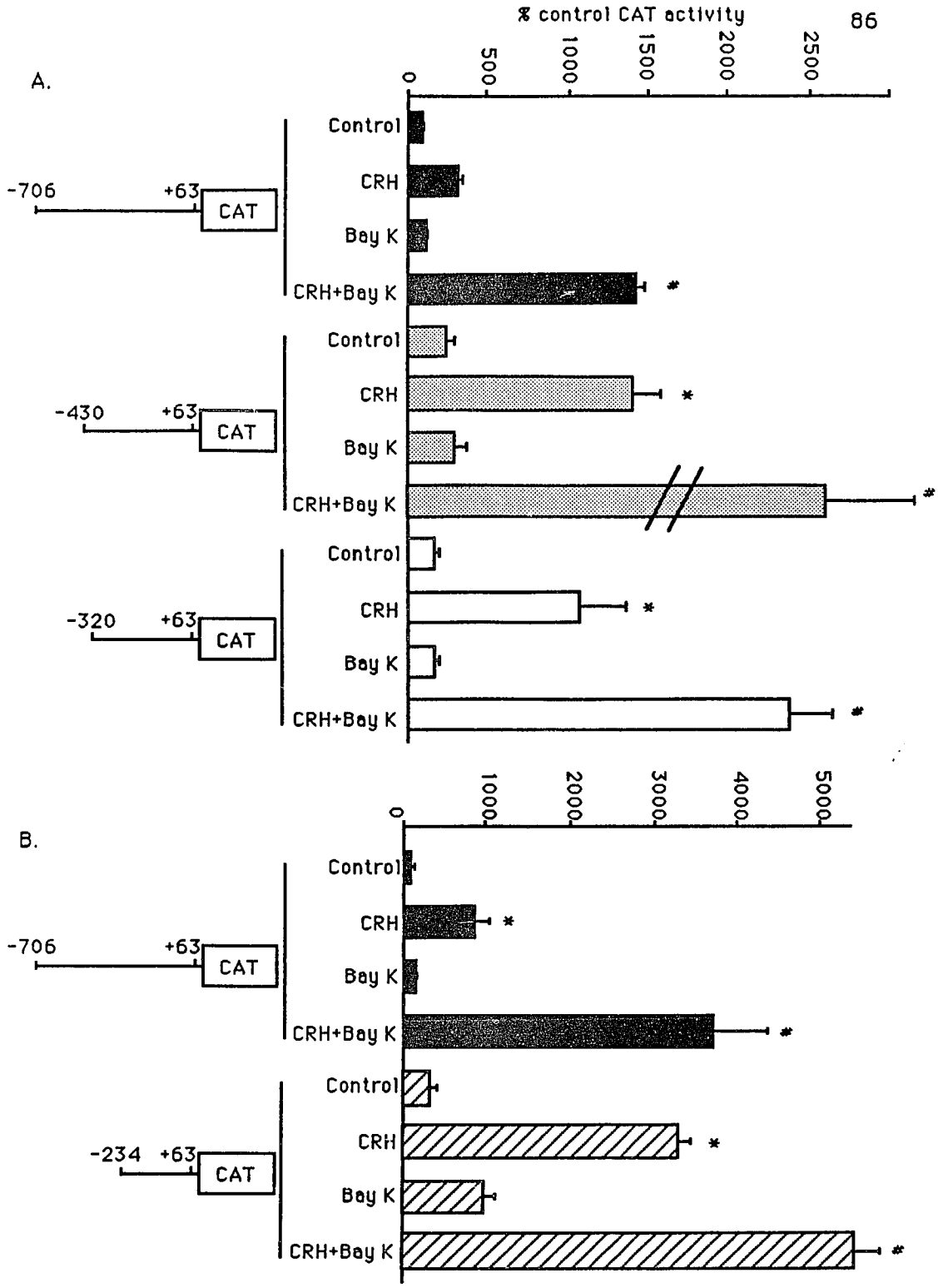


Figure 3. The CAT activity of transfected POMC 5' deletion promoter CAT constructs was examined in AtT20 cells treated with 10nM CRH and/or 1μM Bay K for 18 hrs. Cells were transfected with either p706P-CAT (filled bars), p430-CAT (unfilled bars) or p320-CAT vectors (stippled bars) (A) and p706P-CAT (filled bars) or p234-CAT vectors (striped bars) (B).

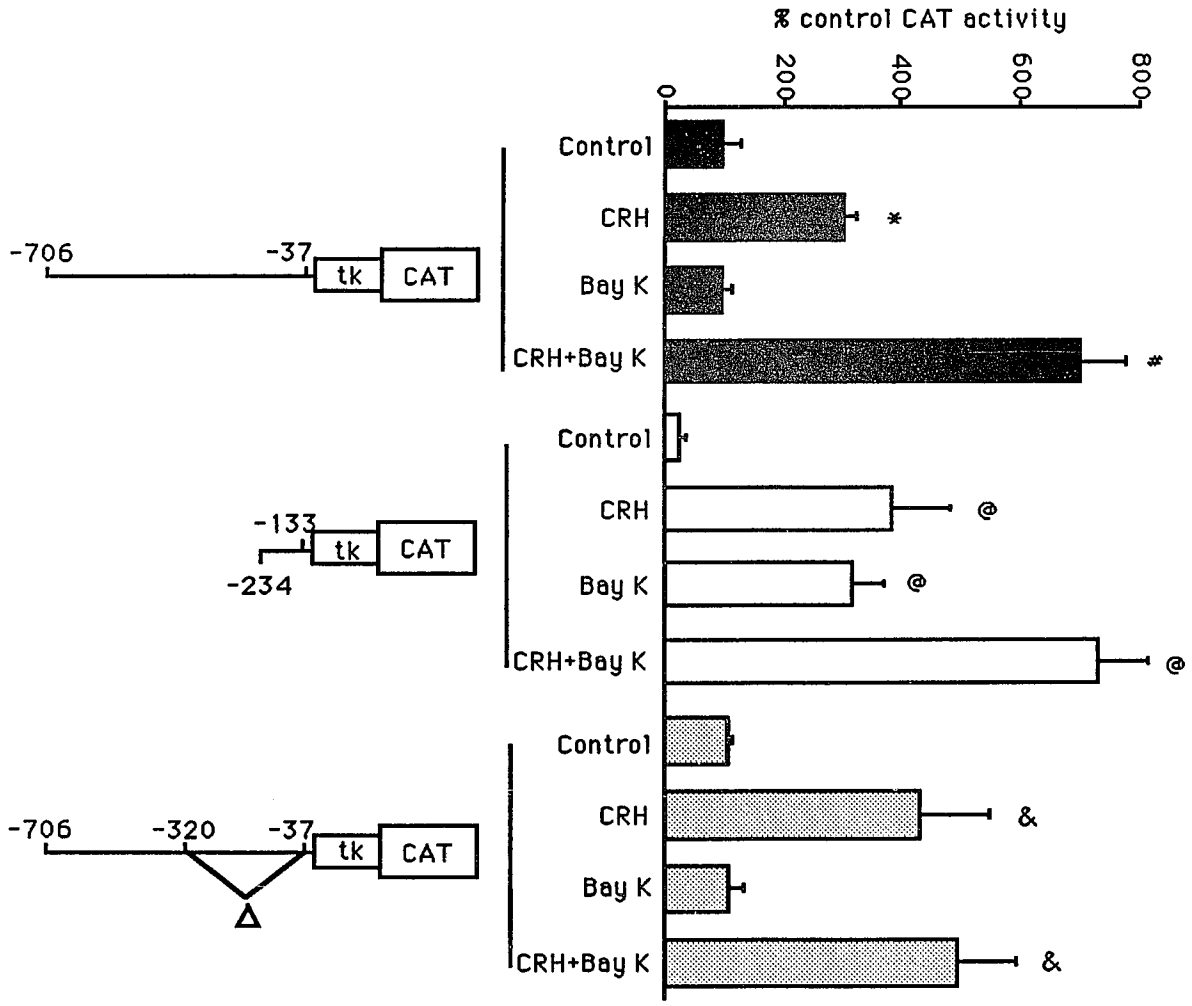
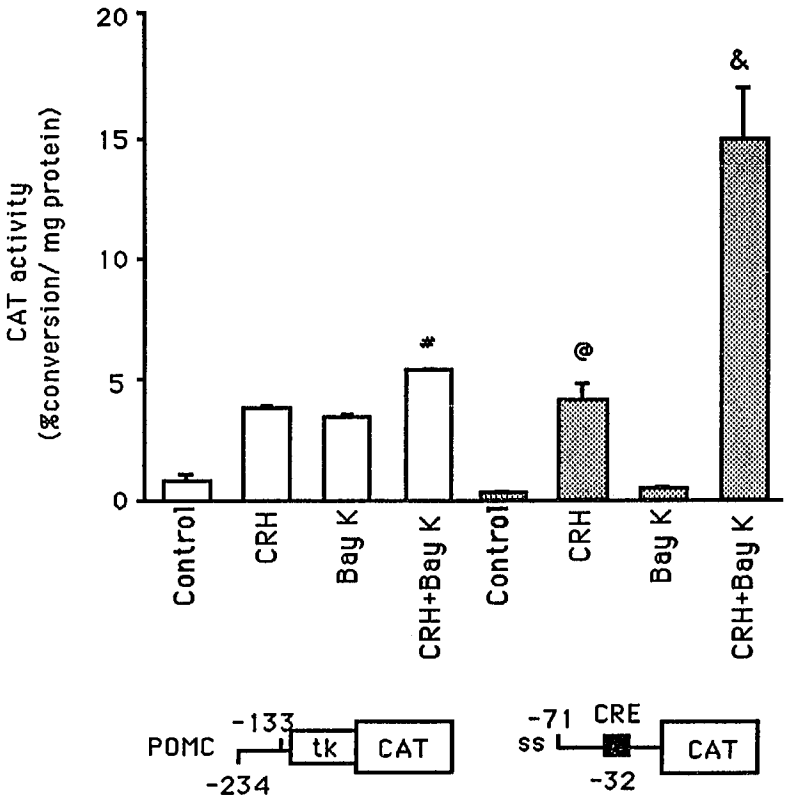


Figure 4. The CAT activity of transfected heterologous promoter fragment constructs was examined in AtT20 cells treated with 10nM CRH and/or 1μM Bay K for 18 hrs. Cells were transfected with either p706P-tkCAT (filled bars), p233/133-tkCAT (unfilled bars) or pΔ320/37-tkCAT vectors (stippled bars). Data is expressed relative to the % CAT activity of untreated p706P-tkCAT-transfected cells measured as CAT conversion relative to milligrams total protein. *, P> 0.05 vs. p706P-tkCAT controls; #, P> 0.05 vs. CRH-treated p706P-tkCAT cells; @, P> 0.05 vs. p233/133-tkCAT controls; &, P> 0.05 vs. pΔ320/37-tkCAT controls;



Figures 5. AtT20 cells were transfected with a heterologous promoter CAT construct containing a fragment of the POMC promoter (-234 to -133 bp) fused to a thymidine kinase promoter driven CAT cassette (unfilled bars) or a somatostatin (ss) promoter-CAT fusion gene containing 71 bp of the 5' termini of the ss 5' flanking sequences relative to the transcription start site, including a consensus CRE element (filled box) at approximately -32 bp (stippled bars). *, P> 0.05 vs. p234/133-CAT controls; @, P> 0.05 vs. ssΔ-71 controls; &, P>0.05 vs. CRH-treated ssΔ-71 cells.

Chapter 5: Conclusion

Understanding the intracellular mechanisms whereby neuropeptide hormones exert their stimulatory effects on gene transcription has been the goal of this thesis project. The mouse anterior pituitary AtT20 D16/16 cell line has provided an excellent corticotrope model system for measuring the acute regulatory effects of peptide and steriod hormones on POMC gene expression. By measuring transcription rate in a transcription run-on assay and the level of primary RNA transcript and processing intermediate accumulation in a solution hybridization/ nuclease protection assay, we have extensively analyzed the acute regulation of POMC gene transcription by the polypeptide hormone CRH. Unlike previous reports which infer mechanisms of POMC gene regulation by measuring changes in POMC mRNA levels or POMC-derived peptide synthesis and secretion at a time when transcriptional events have already been attenuated, the techniques described in this paper measure rapid changes in POMC gene expression occuring shortly (< 60 min) after treatment with hormones or pharmacological agents enabling us to dissect the second messenger pathways mediating neuropeptide regulation. The initial studies demonstrate that POMC gene expression in AtT20 cells is rapidly and differentially regulated at the level of transcription by CRH and Dex in a manner similar to that observed in anterior pituitary corticotropes. Therefore, the AtT-20 cells are an excellent cell model for investigating the regulation of POMC transcription.

In contrast to the stimulatory effects of AVP or PKC activation on POMC derived-peptide release, we demonstrate that short-term treatment with phorbol esters does not activate transcription of the endogenous POMC gene or the 769 bp fragment of the rat POMC promoter in gene transfer experiments. These results are supported by the recent work of Levin et al. (1989) demonstrating the inability of AVP to potentiate CRH-stimulated POMC primary RNA transcript levels or POMC cytoplasmic mRNA levels, despite its ability to augment CRH-evoked ACTH release in anterior pituitary primary culture. Activating the PKC-linked signal transduction pathway may therefore involve posttranscriptional effects on POMC gene expression in AtT20 cells, but does not appear to play a role in direct transcriptional activation.

Since the CRH regulatory effects on POMC-derived peptide secretion are mediated in part by an elevation in cytosolic Ca^{2+}, we examined the ability of intracellular and extracellular Ca^{2+} alone to regulate POMC gene expression. Acutely elevating cytosolic Ca^{2+} without activating the cAMP second messenger pathway with the calcium ionophore ionomycin or

the DHP agonists Bay K was sufficient for stimulating POMC gene transcription and POMC hnRNA levels in AtT20 cells. Activating both the cAMP and Ca^{2+} signal transduction pathways by acute treatment with CRH/8brcAMP and Bay K resulted in a greater induction of POMC hnRNA levels than treatment with either agent alone, suggesting Ca^{2+} plays a permissive role in cAMP regulation of POMC gene transcription. Further studies demonstrated that intracellular Ca^{2+} alone is sufficient for mediating CRH regulation since POMC primary transcript levels were induced by acute treatment with CRH in the absence of external Ca^{2+} and blocked by intracellular Ca^{2+} chelation with Bapta/AM. Blocking VGCC activity had no effect on basal or CRH-regulated POMC gene transcription or POMC hnRNA levels, underscoring the importance of intracellular Ca^{2+} stores for supporting CRH inducibility. When Ca^{2+} is added back to cells that were previously incubated in extracellular Ca^{2+}-free conditions, POMC hnRNA accumulation is induced to levels as high as with CRH or 8brcAMP treatment alone, indicating Ca^{2+} and not cAMP as the major intracellular mediator. Therefore, it appears that while PKA activation is important for CRH activation of POMC gene transcription, Ca^{2+} is an absolute requirement for this event, establishing the dominant role of Ca^{2+} in this signal transduction pathway.

In addition to the lack of transcriptional regulation by PKC activation, calmodulin antagonists also had no effect on basal or CRH-stimulated POMC hnRNA accumulation. Therefore Ca^{2+} appears to be activating POMC gene transcription via some novel mechanism not associated with the typical Ca^{2+} second messenger pathways involving PKC and CaM kinase. Indeed there may be a Ca^{2+}-sensitive mechanism since intracellular Cd^{2+} inhibited POMC hnRNA induction by CRH. A model representing the POMC gene-expressing AtT-20 cell is shown in figure 1.

To elucidate the POMC promoter elements mediating Ca^{2+} regulation, gene transfer experiments utilizing the POMC 5' flanking sequence, linked to an appropriate marker gene, was introduced into AtT20 cells and examined for transcriptional regulation by CRH/cAMP and Bay K. A relatively small region (-704 to +63 bp) of the POMC 5' flanking region conferred most of the regulatory properties displayed by the endogenous gene, including stimulation by CRH/cAMP and inhibition by glucocorticoids. This suggests that a number of cis-acting elements are present in this region of the promoter, since it continued to be expressed and regulated in a tissue- and hormone-specific fashion.

The inability of Bay K to elicit a regulatory effect on the full-length POMC promoter (-704 to +63 bp) in these gene transfer studies was unexpected in light of the previously described stimulatory effects of Bay K on the transcription of the endogenous POMC gene in AtT20 cells. First, this effect may be the result of a short-lived activation of VGCC by Bay K treatment unlike the maintained elevation in cytosolic cAMP (several hours) with longterm CRH or 8brcAMP treatment. Therefore, if the mobilization of cytoslic Ca^{2+} is highly transient, than the overall concentration of Ca^{2+} over a long period of time would be close to baseline levels and therefore insufficient for inducing POMC promoter activity over controls. Second, the POMC-CAT fusion gene may be missing upstream 5' flanking sequences important for Ca^{2+}-regulation necessary for transcriptional regulation of the endogenous.POMC gene.. And third, there may be inhibitory elements within this promoter region that may block Bay K inducibility. The full-length promoter (-704 to +63 bp), however, is activated by Bay K in the presence of elevated cytosolic cAMP with CRH or 8brcAMP treatment suggesting the Ca^{2+}-linked second messenger pathway may potentiate cAMP signal transduction by increasing the sensitivity of the cAMP-dependent cascade. This Ca^{2+}-elicited potentiation of cAMP may result from cooperative interactions between the two second messenger pathways at the level of PKA phosphorylation or binding of transcription factors to DNA elements.

Deletion analysis and insertion of POMC 5' flanking sequences in the proximity of a heterologous promoter has previously identified at least two regions that confer CRH/cAMP inducibility, a strongly regulated region located between -236 and -133 bp, and a weaker more distal element between -477 and -320 bp relative to the transcription initiation site (Roberts et al., 1987). Our studies demonstrate that one of the previously identified CRH/cAMP regulatory regions (-236 to -133 bp) is also sufficient for activation by Bay K. Further analysis is required to determine whether this region of the POMC promoter contains a CaRE distinct from or identical to the CRH-RE.

The limits of this CRH/cAMP and Bay K regulatory region have been further defined by DNase footprinting analysis using crude AtT20 cell extracts (Lundblad et al., manuscript in preparation). Roberts and colleagues have identified a complex protein binding pattern in the Ca^{2+}/cAMP regulatory region of the POMC promoter, suggesting multiple cis-acting factors regulate transcription; combinations of these factors may be necessary for full promoter activity via the CRH signaling pathway. A major footprint in the Ca^{2+}/cAMP responsive region contains a CG rich sequence similar to the AP-2 binding sites of the hMTIIa and proenkephalin genes. Unlike the bona fide AP-2 element, this AP-2-like

sequence does not confer phorbol ester inducibility on the POMC promoter in AtT20 cells. Furthermore, a synthetic AP-2 site derived from the hMTIIa sequence does not compete for binding activity *in vitro* to this promoter region or for soluble factors *in vivo* as measured in functional assays (Steve Salton, unpublished observation). It is doubtful that AP-2 per se is the mediator of the transcriptional response of the POMC gene to CRH, however confirmation will require the use of purified AP-2 in footprinting studies in order to determine whether CRH responsiveness is dependent upon this sequence element. It is also possible that corticotropes use a novel factor related to AP-2 for mediating CRH-regulated POMC gene expression.

Since the POMC gene does not contain a consensus CRE in its 5' flanking region, the molecular mechanisms by which CRH regulates POMC gene expression via the cAMP signaling pathway remains an open area of investigation. In figure 2, we propose a model indicating the basal and regulatory regions of the POMC 5' flanking sequence. The ability of a 100 bp portion of the POMC promoter to confer an enhanced response to both cAMP and Ca^{2+} suggests that a transacting factor(s) may interacts directly or indirectly with this cis-acting region via activation by both PKA and Ca^{2+}-activated protein kinases. Functional synergism between cAMP and Ca^{2+} with such a model could be explained by the cooperative binding of protein(s) to this region of the promoter.

In conclusion, the specific hormone/second messenger-regulatory properties of the gene are not simply determined by the type of cis-acting regulatory elements contained within its 5' flanking region. Overall promoter design may play a key role in establishing how efficiently a gene will be transcribed under a specified set of biological conditions. The literature has shown that particular combinations of transcriptional elements appear to provide unique types of regulation. For example, eventhough AP-1, CREB and AP-4 bind to the CRE region of the proenkephalin gene, neither of these transacting factors alone is capable of mediating cAMP activation, suggesting sequences surrounding the cAMP-responsive regions are essential for cAMP regulation (Goodman, 1990). Because the POMC gene responds to multiple regulatory cues through several cis-acting elements, the promoter environment must play an especially important role in the regulation of the gene. Future studies will focus on the mechanisms behind the functional interaction between second messenger regulatory elements and other promoter elements. These experiments will probe the involvement and nature of protein-protein contacts that occur along the promoter-regulatory region of a gene and mediate its functional cooperativity. The identification of the cellular phosphoproteins regulated by Ca^{2+}-dependent kinases and the

ionic conductance channels modulated by these phosphorylation events is critical for understanding the mechanism of Ca^{2+}-regulated gene expression.

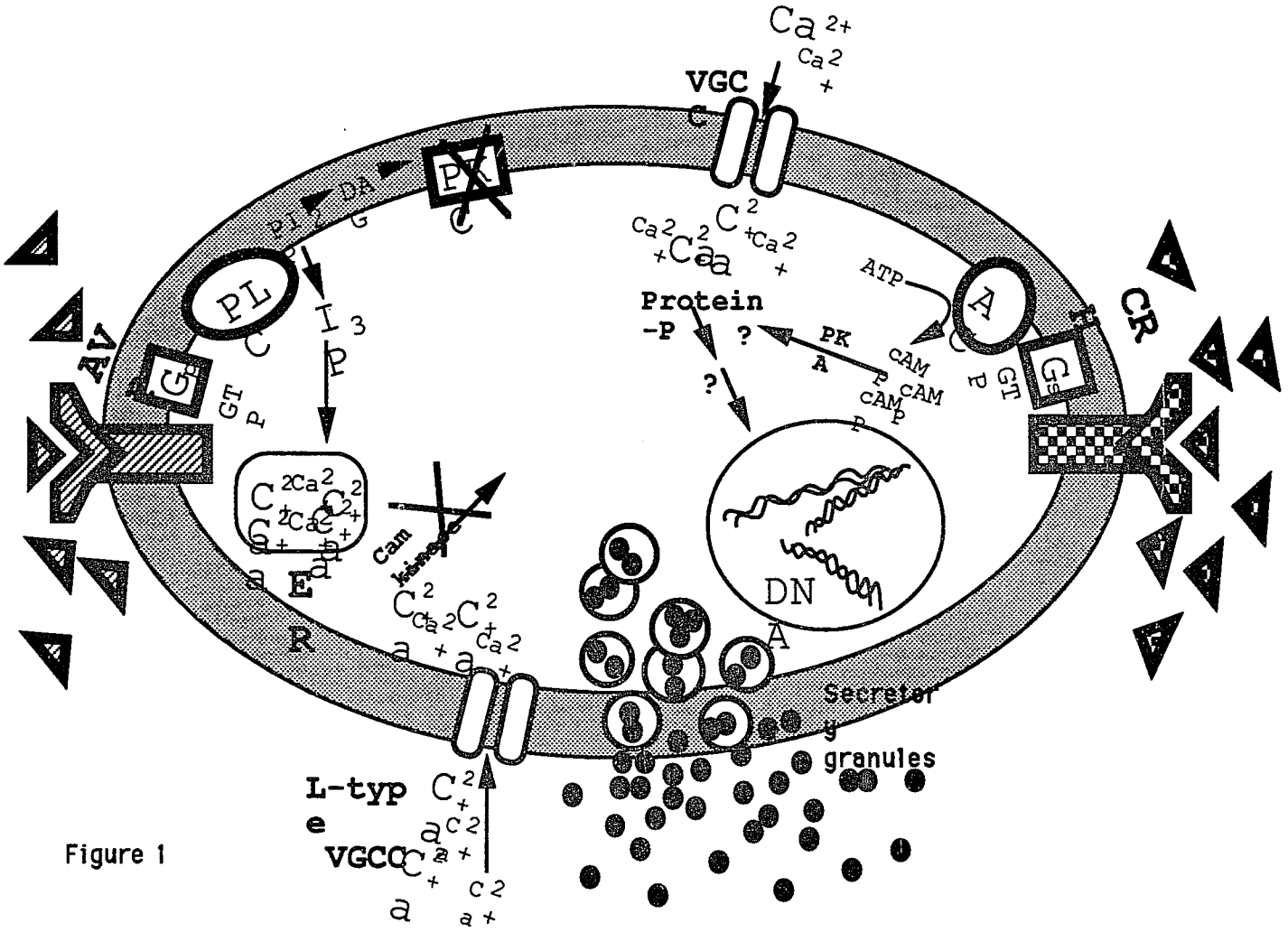

VGC
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Figure 1

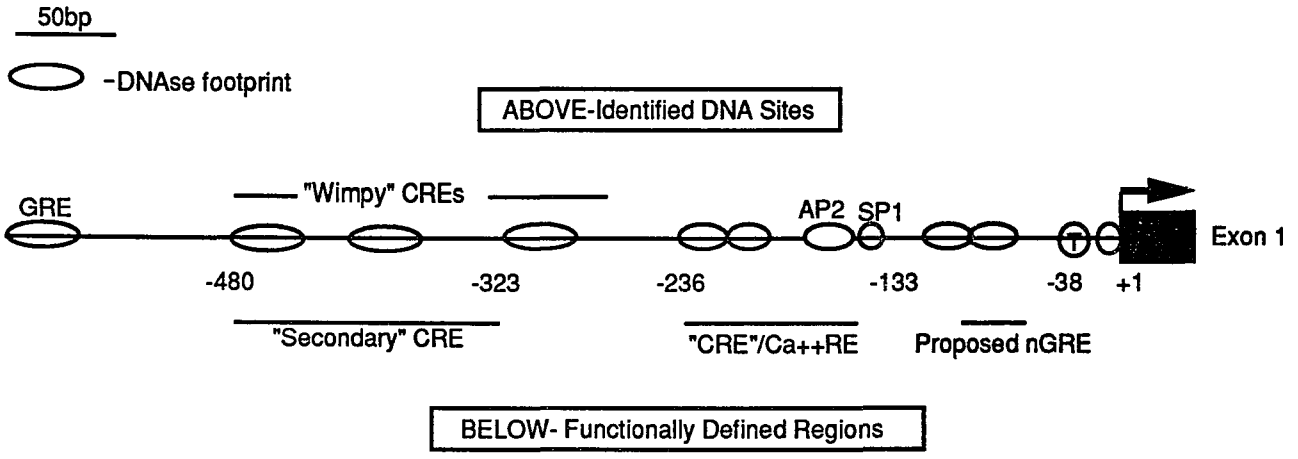

Figure 2. Regulatory regions of the rat POMC 5' flanking sequence

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