Role of Calmodulin-Dependent Protein Kinases and Phosphatase in Calcium-Dependent Transcription of Immediate Early Genes

by

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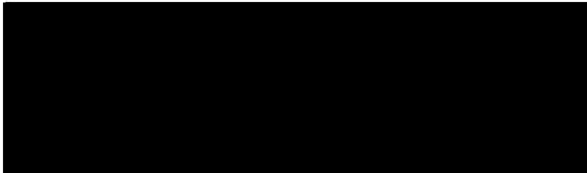

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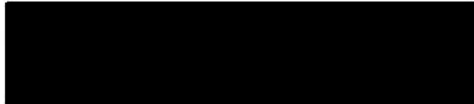

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Abstract

In the nervous system, long-term effects of calcium are due, in part, to the rapid activation of Immediate Early Genes (IEGs) encoding transcription factors. Evidence has emerged during the past few years that calcium regulation of one of these genes, the proto-oncogene *c-fos*, is dependent on the protein calmodulin. Several laboratories have proposed that phosphorylation of the transcription factor CREB by the Ca^{2+}/ CaM-dependent protein kinase II was responsible for the calcium induction of *c-fos*.

Using KN-62, a cell permeable synthetic compound that had been previously characterized as a specific inhibitor of CaM-kinase II, we have examined in PC12 cells the calcium regulation of three IEGs encoding transcription factors: the proto-oncogene c-Fos, the zinc finger protein NGFI-A and the nuclear receptor NGFI-B. KN-62 inhibited by 60 to 80% the calcium induction of all three IEGs. However, KN-62 also inhibited *in vitro* the activity of CaM-kinase IV, another member of the CaM-kinase family. This suggests that both CaM-kinase II and IV could mediate the calcium induction of the three IEGs. CaM-kinase IV is present in the nucleus of neurons, rendering it an attractive candidate for mediating transcriptional regulation by calcium.

We further characterized the ability of CaM-kinase II and CaM-kinase IV to phosphorylate CREB and to activate transcription through phosphorylation of this transcription factor. CaM-kinase II and CaM-kinase IV both phosphorylated CREB at Ser133, the site known to induce transcriptional activation when phosphorylated by PKA. However, only CaM-kinase IV was able to activate transcription through CREB phosphorylation in COS-1 cells. The inability of CaM-kinase II to activate transcription through CREB phosphorylation was postulated to be due to the phosphorylation of another Ser residue, distinct from Ser133. Indeed, this second phosphorylation site has now been

identified as Ser142, and proven to be a dominant negative determinant (1). A specific dephosphorylation of Ser142 leaving Ser133 phosphorylated, would result in the activation of transcription. However, none of the three protein phosphatases tested (protein phosphatases-1, 2A and 2B or calcineurin), preferentially dephosphorylated Ser142 over Ser133.

Calcineurin (CaN), the unique Ca^{2+}/CaM-dependent protein phosphatase, is also involved in the calcium regulation of IEGs. In PC12 cells we used two cell-permeable specific inhibitors of CaN, FK506 and cyclosporin A (CsA), to show that NGFI-A and NGFI-B are negatively and positively regulated by CaN, respectively. However, FK506 or CsA does not affect the induction of *c-fos* by calcium.

These results provide a model in which calcium can regulate transcription of IEGs both positively and negatively. The three IEGs examined in this work can be regulated differently by increases of intracellular calcium depending on the tissue, subcellular location and the respective concentrations of CaN and each CaM-kinase. These observations further suggest that the long-term effects of calcium in the nervous system, through the regulation of IEGs, are mediated both by Ca^{2+}/CaM dependent protein kinases and phosphatase.

I

INTRODUCTION

Eukaryotic cells adapt to chemical, hormonal or electrical changes in their cellular environment by evoking responses such as cellular proliferation, contraction, secretion, metabolic adjustments and changes in gene expression. The intracellular molecules that trigger many of these cellular responses are called second messengers. The three most common second messengers in eucaryotic cells are: 1) the diacylglycerol that, with inositol 1, 4, 5-triphosphate ($InsP_3$), results from the breakdown of PI 4, 5-bisphosphate (PIP_2) by the phosphoinositide-specific phospholipase C (2), 2) cyclic AMP (cAMP) which is the product of the release of pyrophosphate from ATP by adenylate cyclase, and 3) Ca^{2+}, whose intracellular concentration is raised by influx from outside the cell or release from internal organelles (3). Modulation of specific protein kinase and phosphatase activities by these second messengers triggers a cascade of biochemical reactions that ultimately lead to some of the adaptive changes cited above. Approximatively one-third of cellular proteins can be phosphorylated, so modulation of protein kinases and phosphatases constitutes the major mechanism for mediating cellular responses.

Protein kinases and phosphatases can be classified into two main categories depending on whether they phosphorylate/dephosphorylate tyrosines or serines/threonines. Most of the kinases and phosphatases controlled by second messengers belong to the ser/thr family. These kinases can be further divided into multifunctional or substrate specific enzymes. The three best studied multifunctional enzymes are the cAMP dependent-protein kinase (PKA), protein kinase C (PKC) and the Ca^{2+}/CaM-dependent protein kinase II (CaM-kinase II). Diacylglycerol will, with phosphatidylserine and calcium, activate PKC (4, 5); cAMP, by binding to the regulatory subunit of PKA (6,7), will release the active

catalytic subunit of PKA; Ca^{2+}, by forming a complex with calmodulin (one of the major intracellular calcium receptors), will activate a whole family of Ca^{2+}/CaM-dependent protein kinases (8-11) and the protein phosphatase, calcineurin (CaN) (12).

In the nervous system, as well as other tissues, activation of second messenger pathways ultimately results in the modulation of specific gene transcription. In the brain trans-synaptic activity initiates changes that determine the short and long-term phenotypes of neuronal cells. The genes that are responsive to trans-synaptic stimulation in neuronal cells can be classified in two categories: immediate early genes (IEGs) are induced transiently within minutes of stimulation without requiring new protein synthesis (13-17) (see Table 1, p. 5) whereas expression of a second class, the late response genes (17-22), is modified within hours. The modulation of this latter class of genes requires new protein synthesis and is believed to be responsible for long-term changes in neuronal cells. As our work studies the regulation of IEGs transcription, this section will focus on these genes.

IEGs were originally characterized in non-neuronal cells because of their responsiveness to growth factors. The proto-oncogenes *c-fos* and *c-myc* were among the first IEGs identified (23, 24), and they were shown to be very important in the regulation of cell growth as their mutation or deregulation lead to cell transformation. For most IEGs their regulatory function comes from the fact that they regulate the subsequent transcription of other genes. Most IEGs (but not all), such as *c-jun* (25), *c-fos* (26), *c-myc* (27), NGFI-A (28) (also called *zif/268*, *egr-1*, KROX24, Tis8 (29-31, 77)) and NGFI-B (32) (also called *Nur/77*, N10, TIS1, NAK-1 (33-36)) have been shown to encode transcription factors.

The Fos and Jun families of transcription factors are among the best known examples of IEGs whose mechanism of action is well understood (37-40). These two proto-oncogenes encode proteins that interact and function cooperatively as a bimolecular complex in order to regulate the expression of target genes. c-Fos and c-Jun, like other members of the basic region/leucine zipper family of transcription factors (bZip) (41), form a heterodimeric complex through leucine zipper motifs consisting of four to five leucine

TABLE 1. A list of representative Immediate Early genes*

Immediate Early genes	Structure and function
JE	Cytokine
KC, MGSA, gro	Cytokine
β-actin	Cytoskeletal component
2'-5' oligo-adenylate synthetase	Enzyme
Rho-B	Ras-like G protein
c-myc	Transcriptional factor ? Sequences similarities with the Helix loop helix motif of the MyoD E box binding transcription factor family
fos family	Transcription factor, leucine zipper. Members form heterodimeric transcription complexes with members of *jun* family; dimerization occurs through the leucine zipper domain; the complex recognizes the AP-1 consensus sequence -TGACTCA-
c-fos	Function as an activator or repressor depending on *jun* partner
fra-1	Regulatory function unknown
fos-B	Regulatory function unknown
jun family	Transcription factors, leucine zippers. Members form homodimers or heterodimers with other member of the *fos* or *jun* family; dimerization occurs through leucine zipper domain, all complexes recognize the consensus DNA -TGACTCA- sequence
c-jun	c-Fos/c-Jun dimer functions as a transcriptional activator
jun-B	Fos/Jun-B has transcriptional repressor function
jun-D	Constitutively expressed *jun* family member
NGFI-A, *zif/268*, *egr-1*, TIS8, KROX24	Transcription factors, zinc finger containing protein; binds to DNA consensus sequence -GCGGGGGCG-
KROX20, *egr-2*	Transcription factors, zinc finger.
NGFI-B, *Nur/77*, TIS1	Members of the steroid hormone receptor transcription factor family, ligand unknown. Binds DNA consensus sequence -AAAGGTCA- as a monomer
SRF	Transcription factor, binds to the DNA sequence -CC$(AT)_6$GG-

*Adapted from Sheng and Greenberg (1990) Neuron, 4, 477-485

residues regularly spaced at intervals of seven amino acids (41-48). This Fos/Jun complex interacts with the regulatory element known as the transcription factor AP-1 (activator protein-1) binding site 5'-TGAGTCA-3' or the TPA-responsive element (TRE) through basic residues that are adjacent to the leucine zipper motifs and placed in apposition to each other following heterodimerization. The Jun family members can form hetero-and homodimer complexes with each other, while the Fos family members can only form heterodimeric complexes with members of the Jun family (49-52). The fact that different members of each family can form heterodimeric and homodimeric complexes with similar, although not identical, DNA-binding affinities adds another level of complexity in the regulation of transcription through the AP-1 complex. It has also been demonstrated that the AP-1 complexes can interact with cAMP-responsive elements (CRE) 5'-TGACGTCA-3' (50, 53).

Transcriptional activation by c-Fos and c-Jun is regulated by transcriptional, post-transcriptional and post-translational mechanisms. The mRNAs produced by these genes have a very short half-lives, and the termination of their transcription requires new protein synthesis. In the case of the proto-oncogene *c-fos*, destabilizing sequences have been identified in its mRNA, and removal of these sequences increases the stability of the *fos* mRNA (54). Fos and Jun activity are also regulated by protein phosphorylation. Phosphorylation of Ser 361 of Fos by PKA is required for its ability to act as a repressor of its own expression (55-56), and activation of PKC results in the decreased phosphorylation of Jun at sites that negatively regulate its DNA-binding activity (57).

The IEGs NGFI-A and NGFI-B were identified by differential hybridization screening of a cDNA library constructed from PC12 cells treated with NGF and cycloheximide (28, 32). The product of the NGFI-A gene is localized in the nucleus. The NGFI-A protein contains zinc-finger domains, binds specifically to the consensus sequence 5'-GCGGGGGCG-3' and transactivates a promoter containing this sequence (58-60). Binding sites for NGFI-A have been found in many genes such as *jun D* , *c-Ha-ras* (61),

int-2 (62), human *c-abl* (63), rat *hsc73* (64), human histone H3.3 (65), mouse metallothionein (66), the A chain of platelet-derived growth factor (67), the mouse neurofilament protein (68), the murine adenosine deaminase (69), the rat cardiac alpha myosin-heavy chain (70), synapsin I (71), thymidine kinase (72) and NGFI-B (73, 74). In transactivation assays NGFI-A has been shown to activate a reporter gene under the control of promoter regions from synapsin I (75), NGFI-B (74) and thymidine kinase (72). NGFI-A is induced by cAMP, activation of PKC and calcium. NGFI-A has also been identified as a growth response gene (15, 30, 76-80), and it has been established that its product is essential for and restricts differentiation of myeloblasts along the macrophage lineage (81). In the hippocampal model of long term potentiation NGFI-A is induced by high frequency stimulation, and this induction is blocked by antagonists of the Ca^{2+} permeable NMDA-receptor (82, 83), suggesting a role in synaptic plasticity. In support of this model, NGFI-A has recently been identified as a candidate plasticity-related gene using a differential complementary cDNA cloning procedure (84). In addition, chemically and electrically induced seizures activate NGFI-A gene expression (85, 86).

The 60 kDa protein product of NGFI-B is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. NGFI-B has been called an orphan receptor because no ligand has been found for this receptor. However, when synthesized, NGFI-B is constitutively active under all culture conditions examined (87). The transcription of NGFI-B is activated by cAMP, PKC, and calcium in cell culture and in rat brain after seizure induction (88). NGFI-B binds as a monomer to the DNA sequence 5'-AAAGGTCA-3' (NBRE) that contains only a single estrogen receptor "half-site" in contrast to other nuclear receptors that commonly bind as dimers (89). Such DNA binding sites for NGFI-B can be found in the promoter of many genes including those encoding the corticotropin-releasing factor (90-92), lipid binding proteins (93-99), and neuron-related genes including the γ subunit of NGF, the γ subunit of the muscle acetylcholine receptor and N-myc (100-102). An NBRE-like sequence present in the promoter of the steroid

biosynthetic enzyme 21-hydroxylase has been shown to be responsible for the induction of this gene by the hormone ACTH (103). It has very recently been demonstrated by two different laboratories that in T cells NGFI-B is required for apoptotic signals delivered through the T-cell receptor (104-105).

It has been postulated that the products of some IEGs play a critical role in modulating neuronal responsiveness or plasticity. Numerous IEGs encode, as described above, transcription factors which, by regulating the induction of late genes, mediate many long-term responses to trans-synaptic signals in neuronal cells (106-108). The approach to understanding synaptic-regulated gene expression should be at three different levels (109) : (i) how does activation of second messenger pathways regulate IEGs expression; (ii) how do the products of the IEGs regulate the expression of the late genes; and (3) what is the role of IEGs and late genes in the long-term responses of neurons. It has been known for a long time that synaptic activation causes rapid responses in neurons. These responses, which are dictated by the particular stimulation paradigm, vary from milliseconds to minutes depending on the readout parameter. However, more recently, it has also been shown that these short-term stimuli can be transduced to slower, long-term responses in neuronal cells (110, 111). Gene expression is one of the mechanisms that mediate these changes (112).

The focus of our laboratory is to understand the mechanisms of regulation, and the functions of Ca^{2+}/CaM-dependent protein kinases and phosphatase in the nervous system. More particularly, the work of this thesis has focused on the role of CaM-dependent protein kinases and phosphatase in the regulation of IEGs expression by Ca^{2+}. We have decided to focus on the regulation of IEGs expression by calcium because of its critical regulatory role in neuronal cells. Calcium is particularly important in the nervous system where it plays an important role in the organization of the cytoskeleton (113), neuronal excitatibility, synaptic plasticity (114) and learning and memory (115). Ca^{2+} is also one of the major regulators of neurotransmitter synthesis and secretion (116). Neither Na^{+} influx nor K^{+} efflux is required for synaptic transmission, only calcium entering the cell through voltage-dependent

channels in the presynaptic terminal is essential (117). We chose PC12 cells as our model, rather than true neuronal cells, because of the extensive and numerous studies on transcriptional regulation already reported in this cell line.

The basal cytosolic concentration of free calcium in resting cells is 10-100 nM, whereas outside the cell [Ca^{2+}] is in the low mM range. The intracellular calcium concentration rises rapidly to 10^{-6} M or more in response to appropriate incoming signals. Calcium flux through the plasma membrane or the endoplasmic/sarcoplasmic reticulum is regulated by a complex network of exchangers, channels and pumps. This network is responsible for the induction, propagation and termination of the calcium signal. In contrast to the relatively few mediators for cAMP and diacylglycerol, intracellular response to calcium is mediated by a variety of proteins and enzymes. After an increase of intracellular [Ca^{2+}], transmission of the Ca^{2+} signal requires intracellular Ca^{2+} receptors in order to trigger a cellular response. Many Ca^{2+} receptors share in general a common Ca^{2+}-binding motif, a 29 amino-acid helix-loop-helix structure called an EF hand (118). The EF hands bind Ca^{2+} with K_ds ranging from 10^{-9} M (parvalbumin) to 10^{-5} M (low affinity sites of calmodulin). Upon binding of calcium, these intracellular receptors undergo conformational changes which allow their interaction with and activation of target proteins.

Among the increasing number of Ca^{2+} binding proteins being identified in the brain, calmodulin is unique because it interacts and controls the activity of many proteins such as NO synthetase (119-121), adenylate cyclase (122), plasma membrane ATPase (123), inositol 1, 4, 5-triphosphate (IP_3) kinase (124), CaM-kinase I (125), II (8), III (126, 127) and IV (128-130) as well as the unique Ca^{2+}/CaM-dependent protein phosphatase calcineurin (12). However, in the brain as well as in other tissues, the major CaM-binding proteins are the CaM-kinase II and calcineurin, each of which constitutes 0.5-1.0% of total brain protein.

The biological role of calcium in the nucleus has not received a lot of attention. This is surprising because it has long been known that Ca^{2+} is important in the regulation of nuclear functions. The observation that small peptides freely diffuse from the cytosol into the nuclei lead to the believe that nuclei were incapable to maintain ion gradients accross their membranes. However, the recent report of the presence of selective ion channels within the nuclear membrane (131) demonstrated that it was not the case. Conflicting results have been reported concerning stimulation-induced changes of free-calcium in the nucleus of neurons (132). Using fluorescence imaging techniques different response of nucleoplasmic and cytosolic Ca^{2+} concentrations in response to extracellular stimuli have been reported in a variety of different cell types (133-136). Some studies have demonstrated that isolated nuclei are capable of ATP-dependent Ca^{2+}-uptake and IP_3-inducible Ca^{2+} release (137, 138). It is now clear that Ca^{2+} and Ca^{2+}-binding proteins regulate both the structure of the nucleus (i.e., during cell cycle progression (139)) and the function of the nucleus (i.e., gene transcription, see below). The Ca^{2+}-binding protein CaM is present in the nucleus of neurons as well as other cell types (140-144). It has now been clearly demonstrated that the nuclear CaM levels are modulated by the action of hormones and can be modified by different pharmacological agents (145). CaM as well as other Ca^{2+}-binding proteins such as the protease, calpain, are clearly involved in the regulation of nuclear events (146).

A. *Regulation of gene expression by calcium*

The role of calcium and calmodulin in the regulation of gene expression has now been reported for numerous genes, including prolactin (147-149), proopiomelanocortin (150), insulin (151), tyrosine hydroxylase (152), muscarinic receptor (153), nicotinic acetylcholine receptor (154) and the IEGs *c-fos* (155-159), NGFI-A (16) and NGFI-B (16, 73). Our knowledge of transcriptional regulation by calcium progressed mainly because of the results obtained in the nervous and the immune systems within the past 5 to 8 years.

In PC12 cells the transcription of many IEGs is strongly increased upon stimulation by various neurotransmitters, growth factors and membrane depolarization with sometimes markedly different responses (16). In regards to the effects of calcium, Morgan and Curran demonstrated in 1986 for the first time that *c-fos* induction in PC12 cells by KCl, veratridine (depolarization *via* Na^+ channel activation) and BK8644 (calcium channel agonist), but not the induction by NGF, all depended on extracellular calcium and were blocked by several CaM antagonists (e.g., chlorpromazine, trifluoperazine and W7) (14). These results were particularly interesting since induction of *c-fos* by the phorbol ester PMA was not affected by CaM antagonists, suggesting that a calmodulin -mediated modification of a transcriptional activating protein (TAP) was responsible for the induction of *c-fos* by calcium.

Transcriptional regulation by Ca^{2+}/CaM-dependent protein kinases

The best studied Ca^{2+}-responsive cellular IEG involved in signal transduction is *c-fos*, and this gene has recently attracted a lot of attention from neurobiologists. One of the most successful strategies has been to borrow concepts and reagents from oncogene research and to apply them to the nervous system (37). Since the report by Morgan *et al.* in 1986, the mechanism(s) by which calcium control *c-fos* expression has been extended principally through the laboratory of Michael Greenberg. These investigators have identified

a calcium responsive element (CaRE) about 60 bp 5' of the mRNA start site in human *c-fos* (155, 156). The sequence of the CaRE is very similar to the consensus CRE which had been identified as a cAMP-responsive element in the promoter of the somatostatin gene (160) and of many other cAMP responsive genes (161). The CaRE sequence also mediates cAMP induction of *c-fos* (162), and mutational analyses of this sequence showed identical bases are essentials for both calcium and cAMP induction (156). However, this sequence is not involved in the activation of *c-fos* by serum and growth factors . The transcription factor CREB (see Figure 1, p.13), which binds to the CaRE/CRE, mediates Ca^{2+}-inducibility by membrane depolarization/ionophore treatment. In fact, upon membrane depolarization CREB is phosphorylated (independently of a cAMP-activated pathway) (156, 157) on the site (Ser133) known to be responsible for transcriptional activation by PKA (163) (see Figure 1, p.13). In PC12 cells, expression of a construct in which CREB had been fused to the DNA binding domain of the yeast transcriptional activator Gal4 co-transfected with a reporter gene under the control of Gal4 binding sites allowed the specific analysis of transactivation by CREB without interference from endogenous CRE-binding proteins. In this system, CREB was shown to function as a Ca^{2+}-regulated transcription factor upon membrane depolarization or treatment with the calcium ionophore A_{23187}, and mutation of Ser133 abolished the ability of CREB to mediate activation by Ca^{2+}(157). However, evidence that calcium and cAMP converge on the CaRE-CRE/CREB system is further strenghtened by the fact that calcium and cAMP act synergistically to induce transcription through the CaRE *in vitro*, (164) in PC12 cells (156) and in pancreatic islet cells (165). Other members of the CREB family of transcription factors, the Activating Transcription Factor-1 (ATF-1) and the CRE modulator CREM can also mediate calcium and cAMP signals although with some differences as compared to CREB (166, 248).

CaM-kinase II has been proposed by a number of laboratories (157, 164, 165) to mediate CREB phosphorylation upon an increase of intracellular calcium since i) it phosphorylates CREB on Ser133 *in vitro* (157, 164) (see Figure 1, p.13), ii) *in vitro*

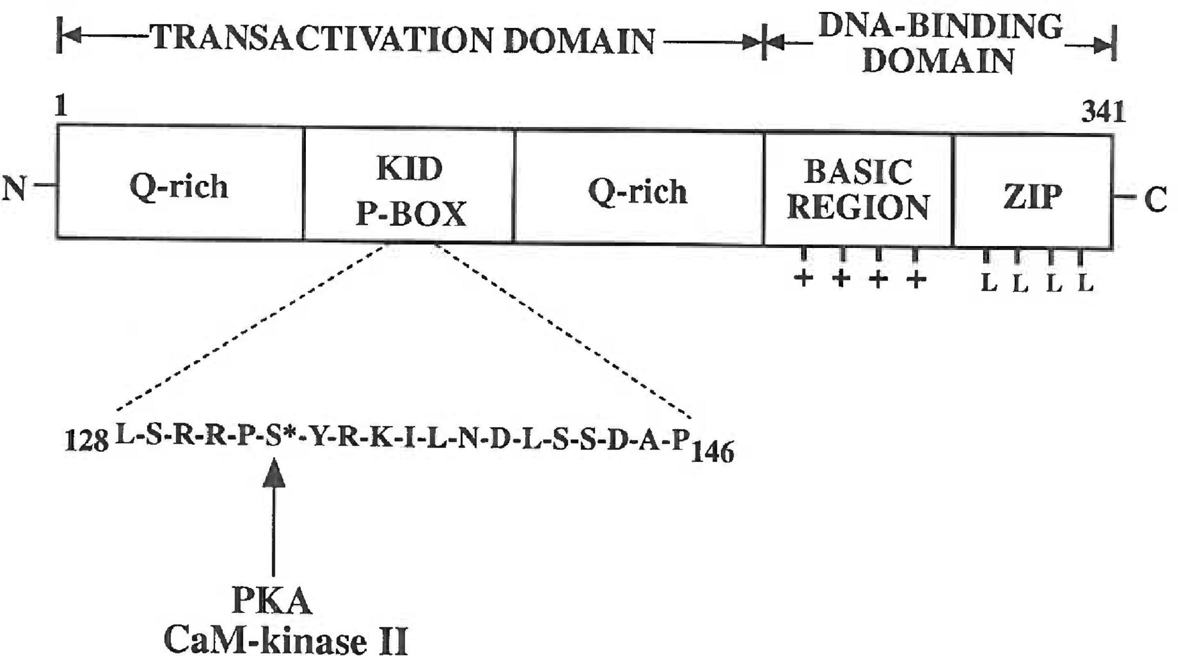

Figure 1. Structure of CREB#. Detailed is the amino-acid sequence of the region of the kinase-inducible domain (KID) or phosphorylation box (P-BOX) that contains serine-133 (*) the site that triggers CREB activation when phosphorylated by PKA (and CaM-kinase II *in vitro*). Additional serines can be phosphorylated by other kinases, such as CKII and GSK-3 kinase, N and C-terminal of serine-133. The phosphorylation of KID is believed to allosterically change the conformation of the transactivation domain bringing the glutamine-rich (Q-rich) regions flanking KID into an active conformation for positive interactions with the TATA box complex of factors involved in initiation of transcription. The DNA-binding region of CREB consists of the basic region and the leucine zipper (ZIP).

Adapted from Meyer and Habener, (1993) *Endocrinol. Rev.*, 14 (3), 269-290

transcription assays using a CaRE-containing *c-fos* promoter indicated that phosphorylation of CREB by CaM-kinase II results in a small increase in gene transcription (164) and iii) KN-62, a cell permeable compound that has been originally characterized as a specific inhibitor of CaM-kinase II (379), blocked membrane depolarized-activation of a reporter under the control of the glucagon CRE (165) or the *c-fos* CaRE sequence (158). This hypothesis is supported by the fact that CaM-kinase II is abundant in PC12 cells and it is activated by membrane depolarization (167) or release of calcium from intracellular stores (168). However, no stimulation of a CRE-reporter gene was observed in pituitary cells co-transfected with an expression vector encoding a constitutively-active form of CaM-kinase II (169). The same constitutively-active form of CaM-kinase II gives a 60-fold stimulation of expression of a reporter gene under the control of a region of the Rous Sarcoma Virus Long Terminal Repeat (RSV LTR) (169). This region, called the CaM-kinase-response element (CaMRE), binds the bZip transcription factor C/EBPβ (also called NFIL-6, AGP/EBP, LAP, IL6/DBP (170-173)). CaM-kinase II phosphorylates C/EBPß within the leucine zipper at position 276, and KN-62 prevents the phosphorylation of C/EBPβ in GH3 cells upon treatment with the calcium ionophore A_{23187} (174). Mutation of Ser276 to Ala abolished the ability of the constitutively-active form of CaM-kinase II to activate transcription through the CaMRE (174). Thus, evidence for a role of CaM-kinase II in transcriptional regulation through the C/EBPß system is strong, but CaM-kinase II appears to be quite weak in stimulating transcription *via* the CRE/CREB system. Another CaM-kinase, CaM-kinase I can also phosphorylate CREB at Ser133 *in vitro* (157); however, there is no evidence that this enzyme is involved in the regulation of transcription by calcium and calmodulin.

Transcriptional regulation by the Ca^{2+}/CaM-dependent protein phosphatase: Calcineurin

If calcium-induced phosphorylation can regulate transcription, then calcium induced-dephosphorylation may also be involved in transcriptional regulation. Indeed, it has recently

been demonstrated that calcineurin also regulates gene transcription. Cyclosporin (CsA) (175), and more recently FK506 (176, 177), have been used clinically for years as very potent immunosuppressors without understanding their mode of action at the molecular level. These drugs blocked early events in the activation of T-lymphocytes, including IL-2 gene transcription (178-180). Recently, the intracellular receptors of CsA (181) and FK506 (182, 183) have been characterized as proline isomerases called immunophilins. Calcineurin (CaN) (12), the unique Ca^{2+}/CaM-dependent protein phosphatase, binds to the drug-isomerase complexes (184, 185) with consequent inhibition of its Ca^{2+}-dependent phosphatase activity (184-186). Nonimmunosuppressive cyclophilin-binding cyclosporin that does not inhibit CaN activity has been characterized (187). It is now clear that the inhibition of CaN by the immunosupressor-isomerase complex in T cells (188, 189) is responsible for the inhibition of IL-2 transcription (190, 191). NF-ATp, a 120 kDa protein (192) constitutively phosphorylated in unstimulated T-cells, is part of a complex that binds DNA and is required for IL-2 gene transcription. Two groups have recently found NF-ATp to be a substrate of CaN *in vitro* (193, 194). The kinase that phosphorylates NF-ATp is as yet unknown. The phospho-NF-ATp is restricted to the cytosol, and upon dephosphorylation NF-ATp translocates into the nucleus (193, 195). Activation of IL-2 transcription in Jurkat cells requires not only an increase of intracellular calcium, but also phorbol ester treatment which results in the activation of PKC. In the nucleus NF-ATp interacts with the transcription factors Fos and Jun in order to regulate IL-2 gene transcription (194), thus providing a novel mechanism for regulation of gene transcription which integrates the calcium-dependent and protein kinase C-dependent pathways of T cell activation. In a pancreatic islet cell line, CsA and FK506 inhibit CRE-mediated gene transcription after membrane depolarization (196). Rapamycin antagonizes the inhibitory effect of FK506 but not of CsA, suggesting that FK506 and CsA may act through complex formation with distinct intracellular immunopholins (196). Overexpression of calcineurin renders these cells even more resistant to the inhibitory effects of CsA and FK506 (196).

Additional pathways used by calcium to regulate IEGs

Depending on the cell line or mode of increase, Ca^{2+} can activate different signalling pathways that result in the induction of *c-fos*. In hippocampal neurons both membrane depolarization and stimulation of NMDA receptors result in elevation of intracellular Ca^{2+}, activation of CaM-kinase II (197) and induction of *c-fos* (158). However, induction of *c-fos* by membrane depolarization, but not by NMDA receptor stimulation, is blocked by the CaM-kinase antagonist KN-62. The NMDA response appears to be mediated by the serum response element (SRE), not the CaRE/CRE (158). The SRE can also mediate Ca^{2+}-induction of *c-fos* in response to treatment of fibroblasts with thapsigargin, an inhibitor of the Ca^{2+}/ATPase pump (198). In fibroblasts, macrophages and Myeloid leukemia cells, Ca^{2+} induction of *c-fos* may largely involve relief of a block in transcriptional elongation in the first intron rather than transcriptional initiation (199-201).

The regulation of other IEGs by calcium has also been investigated (16). The most interesting results have been obtained for NGFI-B. The transcription of NGFI-B is activated by growth factors and second messenger pathways such as cAMP and calcium. However, in PC12 cells stimulation of NGFI-B expression by membrane depolarization is 20-fold stronger than by growth factors, even though NGFI-B was initially identified as a NGF-inducible gene. Yoon *et al.* (73) identified a portion of the NGFI-B promoter essential for Ca^{2+} stimulation. This sequence of 64 nucleotides (from -22 to -86) within the NGFI-B promoter is responsible for the induction by membrane depolarization and NGF in PC12 cells (73). Although this sequence does not contain a CRE/CaRE motif, it contains a number of potential binding sites for known transcription factors including two AP-1-like sequences very similar to the FAP element in the *c-fos* promoter which can function as an AP-1 or CRE (202-205), two GC-rich sequences with two overlapping copies of the SP1-binding site (206), and a binding site for a family of zinc-finger proteins including NGFI-A (58, 207). In fibroblasts, this latter region of the NGFI-B promoter centered at -55, has

been shown to bind NGFI-A after serum stimulation. In addition, an expression vector encoding NGFI-A transactivates a reporter gene under the control of the first 126 nucleotides of the NGFI-B promoter (74). This result is particularly interesting due to the fact that this region (-22 to -86) of the promoter also confers a delayed-early expression of NGFI-B to serum in fibroblasts (74). In fact, although initially believed to be a serum-inducible immediate-early gene (77), transcriptional activation of NGFI-B by serum in fibroblasts is composed of two components; an immediate-early one, which can occur in the absence of *de novo* protein synthesis, and a delayed-early component, which is dependent on *de novo* protein synthesis (possibly NGFI-A). The immediate early expression of NGFI-B by serum is mediated primarily by sequences located between nucleotides -86 and -126 upstream of the transcription start site. However induction of NGFI-B by NGF or membrane depolarization in PC12 cells does not require *de novo* protein synthesis, is mediated by the region -22 to -86 and shows no evidence of a delayed-early component (74).

In contrast to NGFI-B, NGFI-A is induced to 5-fold greater levels by growth factors than by depolarization. The regulation of NGFI-A by calcium is difficult to investigate due to its weaker responsiveness in comparison to *c-fos* and NGFI-B. Thus, we don't know which promoter sequence(s) in NGFI-A are responsible for Ca^{2+} induction. The promoter of NGFI-A contains (at positions -84, -106, -370 and -408) four sequences similar to the serum-response-element (SRE), a GC-box (the recognition site for the transcription factor Sp1 (208)) centered at -286 and a sequence related to the CRE located at nucleotide -140 (209). Multiple signal transduction pathways converge on the same sequences of the NGFI-A promoter to regulate the expression of this gene. Two SREs and an AP-1-like sequence present within the first 106 bp of the NGFI-A promoter are necessary for induction of this IEG by NGF, TPA and serum in PC12 cells, and any two of the three sequences described above are sufficient for induction by NGF (210).

In some cases activation of a Ca^{2+}/CaM-dependent protein kinase or phosphatase cannot account for the results reported on gene induction by calcium, and cross-talk between the calcium and other signalling pathways have been described. For example, inhibition of PKA abolishes the ability of calcium to activate the transcription of *c-fos* (211, 212) and NGFI-A in PC12 cells (211). Calcium can regulate the level of intracellular cAMP through CaM-dependent adenylate cyclase which has recently been cloned and characterized (122). The cross-talk between the calcium and the cAMP signalling pathways can also occur at the level of PKA. A model has been proposed whereby Ca^{2+} and cAMP converge directly on PKA (213). In this model Ca^{2+} activates the protease calpain which may degrade the regulatory subunit of PKA and thereby lead to increased levels of its active catalytic subunit. This model predicts that Ca^{2+} and cAMP converge to produce a prolonged increase in PKA activity, greater than cAMP alone. Further studies will be necessary to understand the role of PKA in the regulation of gene expression by calcium. However, it is interesting to note that calpain has been reported to modulate gene transcription by regulating the cellular levels of transcription factor proteins such as c-Fos and c-Jun, possibly through removal of the DNA-binding domain (214, 215).

B. ***Role of phosphorylation in the regulation of gene expression***

Phosphorylation regulates transcription factor activity at three different levels (216). The transcription factor or its protein kinase can be sequestered in the cytosol and thus be inactive because it cannot reach its target in the nucleus. Activation of the kinase or phosphorylation (dephosphorylation if the transcription factor is constitutively-phosphorylated) of the transcription factor or its cytoplasmic anchoring protein allows translocation into the nucleus. Secondly, phosphorylation of the transcription factor modulates its binding (positively or negatively) to target DNA. Lastly, interaction of the transcription factor with the transcriptional machinery can be modified by phosphorylation of the transcription factor within its transactivation domain. These three different types of regulation are not exclusive and each transcription factor can be regulated by phosphorylation at several distincts levels.

Regulation of transcription factors by nuclear translocation

Regulation of transcription by compartmentalization is limited to eukaryotes as prokaryotes do not have nuclei. The main examples of such regulation are the transcription factors SWI5 (217, 218), ISGF3 (219, 220), NF-ATp (see above), the Rel-related family of transcription factors (NF-κB p50, NF-κB p65, p49, c-Rel, Rel B and Dorsal) (221) (222) and the CAAT-enhancer binding protein β (C/EBPβ) (170-173). In unstimulated cells, except in B cells, NF-κB is held in the cytoplasm in an inactive complex with the inhibitory protein IκB (223). In response to various stimuli such as TPA, the complex NF-κB/IκB dissociates (224), probably due to phosphorylation of IkB (225-228), and NF-kB-DNA binding activity can be detected in the nucleus (223). C/EBPβ (see above) localization varies from cell to cell. It is mainly localized in the nucleus of Hela and G/C cells (173, 174) but is cytosolic in unstimulated PC12 cells (173). In PC12 cells, C/EBPβ is induced to translocate from the cytosol to the nucleus by an increase of the intracellular concentration of

cAMP (173); however, there is no evidence that C/EBPβ is a direct substrate of PKA in cells.

Regulation of DNA binding activity

Transcription factor binding activity can be affected either positively or negatively by phosphorylation. Phosphorylation-induced negative regulation of transcription factor binding activity has been demonstrated for c-Myb, c-Jun, the basic helix-loop-helix (bHLH) protein-Max, myogenin and the POU proteins, Oct1 and GHF1/Pit1. Phosphorylation of c-Myb by casein kinase II (CKII) results in a large decrease in DNA binding activity which is reversed by phosphatase treatment (229). The proto-oncogene c-Jun is phosphorylated *in vitro* in its C-terminal domain by several protein kinases including GSK-3, casein kinase II, ERT, and cyclin B/p34^{cdc2}. However, only phosphorylation by GSK-3 and CKII has been shown to inhibit c-Jun DNA binding (57, 230, 231). This inhibition can be reversed by phosphatase treatment (231, 232). The inhibitory sites are dephosphorylated in cells after activation of PKC or expression of several oncogenes such as *v-Ha-ras*, *v-sis* and *v-src* (232-234), probably by activation of a protein phosphatase. Max and myogenin DNA binding activity are inhibited after phosphorylation by CK II and PKC, respectively (235, 236). PKA phosphorylation of the POU proteins Oct1 and GHF1/Pit1 inhibits their abilities to bind DNA (237-238).

The best studied transcription factor whose DNA binding is increased upon phosphorylation is the serum response factor (SRF). SRF is phosphorylated *in vivo* on many serine residues (239-242). Phosphorylation of recombinant SRF by CK II stimulates DNA binding (241, 242). However, the activation of CK II by growth factors is highly controversial (243, 244). One of the sites phosphorylated, Ser 103, which is required for maximal DNA binding activity *in vitro,* is contained within a consensus phosphorylation sequence for PKA (241).

Modulation of DNA binding by phosphorylation can occur through different mechanisms. In the case of positive regulation several mechanisms are possible. As described above phosphorylation by CKII of the SRF enhances its DNA-binding activity. Phosphorylation occurs at or around residue 83 and affect the activity of the DNA-binding domain located 50 amino-acids away (amino-acids 133 to 264). Three different mechanisms that could explain such effects have been tested (240). First, phosphorylation might have affected SRF dimerization which is required for SRF's DNA-binding activity, however phosphorylation of the dimers did not have any effect on dimerization. Second, the amino-terminal domain of the SRF might mask the DNA-binding domain, thus inhibiting its activity. This inhibition could have been relieved by phosphorylation, but deletion of the amino-terminal domain did not result in elevated DNA binding activity. A third possibility is that phosphorylation altered the conformation of SRF, resulting in increased DNA-binding activity. To test this possibility, SRF's structure was probed by partial tryptic digestion. Changes in the preferred sites of cleavage were observed after phosphorylation of SRF. These altered sites have been localized to the region of the DNA-binding domain. Therefore phosphorylation of SRF at Ser83 appears to cause a conformational change in the DNA-binding domain over 50 amino acids away (240). In the case of negative regulation, usually the site phosphorylated is located within or near the DNA binding domain, and phosphorylation interferes with DNA binding by electrostatic repulsion between the phospho-groups of the protein and phosphates on the DNA. When the phosphorylation site is located far away from the DNA binding domain, it is likely that phosphorylation alters the conformation and/or dimerization of the protein, thus altering its ability to bind to DNA.

Regulation of transcription factor transactivation

A certain number of transcription factors are regulated by phosphorylation-induced transactivation, and I will mainly focus this section on the cAMP responsive element binding protein CREB (see Figure 1, p.13). Since the identification (245) and the cloning

(246, 247) of CREB as the protein that binds the CRE/CaRE sequence, a whole family of CREB transcription factors has been uncovered (248). Some members of this family are transcriptional activators (CREB, CREMτ and ATF-1) (249), while others are transcriptional repressors (CREM α, β and γ; and the CREM generated products ICER, E4BP4 and CREB-2 (250-253). This part of the introduction will focus on the transcriptional activators of the CREB family and particularly on CREB.

The importance of CREB in cAMP-mediated regulation of gene expression has been clearly established (161, 249, 248). Upon binding of cAMP, PKA dissociates into a regulatory subunit dimer and two free catalytic subunits. The catalytic subunit translocates to the nucleus where it phosphorylates nuclear proteins including CREB, CREM τ and ATF-1. Two major protein isoforms of CREB have been cloned, CREB327 (327 amino-acids) (254) and CREB 341 (341 amino-acids) (247). The two isoforms are encoded from the same gene by two alternatively spliced mRNAs. The relative effectiveness of transactivation by each isomer remains controversial (255-257). CREB, like c-Fos and c-Jun, is a member of the dimeric bZip family of transcription factors (41). CREB is highly conserved within mamalian species and preferentially forms homodimers, although, it can form heterodimers with some other members of the bZip family.

CREB (see Figure 1, p.13) is composed of two major domains, a transactivation domain composing about three-fourths of the protein and located in the N-terminus, and the DNA-binding domain located in the C-terminus. The transactivation domain has been extensively analyzed (247, 255) and can be divided into three subdomains, a kinase inducible domain (KID) that contains the major phosphorylation sites, and two glutamine-rich regions (Q-rich) flanking the KID domain. The KID domain is approximatively 50 amino-acids long. It contains numerous serine as well as acidic residues appropriately arranged such that the serines (and threonines) are phosphorylated by PKA and possibly other protein kinases such as the CaM-kinases, CKII, PKC and GSK-3 kinase. In fact, phosphorylation by PKA of a single residue, Ser133 in CREB341 (or equivalent Ser119 in

CREB 327), is the initial and key event to generate the transactivation function of CREB (163). Mutation of Ser133 to Ala abolishes transactivation activity (163). It is postulated that the acidic KID region of CREB may be forming a β-sheet structure important for transactivation as demonstrated for the yeast transcriptional activators GAL4 and GCN4 (258). It has not been ruled out that Ser133 phosphorylation triggers a cascade of phosphorylation on adjacent sites by processive or hierarchal kinases such as CKII or GSK-3 kinase (259). One other region carboxy-proximal to Ser133 is critical for transactivation (260). Most experiments suggest that phosphorylation by PKA has no effect on either dimerization or DNA binding activity of CREB measured *in vitro* (261), but phosphorylation of one or more Ser between residues 106 and 122, possibly by CK II, may also be needed for maximum CREB transactivation (262).

CREB activity is also regulated by protein phosphatase(s). After an initial burst of phosphorylation in response to cAMP, CREB is dephosphorylated and transcription of the cAMP responsive somatostatin gene is correspondingly reduced (263). The identity of the protein phosphatase responsible for Ser133 dephosphorylation is still controversial. Two reports implicate protein phosphatase 1(PP1) as the CREB phosphatase (263-264). The phosphatase inhibitor 1 protein and okadaic acid both prevented the dephosphorylation of CREB at Ser133 in PC12 cells and also augmented the transcriptional response to cAMP (263). At the concentrations used in this study, only PP1 appears to be similarly inhibited by these agents. Moreover, microinjection in fibroblasts of an expression vector encoding a constitutively-active form of inhibitor 1 (I-1), a PP1 specific inhibitor, by itself resulted in an apparent increase in phosphorylated CREB in unstimulated cells (264). In addition, injection of the I-1 expression vector activated expression from a coinjected CRE-lacZ reporter plasmid indicating that the increased phosphorylation of CREB also stimulated its transcriptional activity (264). Two other reports from a different laboratory suggest that the phosphatase that dephosphorylates CREB at Ser133 is PP2A (265, 266). In these reports, the P-CREB phosphatase activity present in rat liver nuclear extract coelutes with the

Ser/Thr protein phosphatase type 2A (PP2A) on Mono-Q, amino-hexyl sepharose, and heparin agarose and was chromatographically resolved from nuclear PP1. Furthermore, P-CREB phosphatase activity in nuclear extracts was unaffected by the heat-stable protein phosphatase inhibitor-2, which is a potent and selective inhibitor of PP1 (267). In addition, these investigators showed that Simian virus 40 small tumor antigen, which alters the ability of PP2A to dephosphorylate substrate proteins (268-270), inhibits the dephosphorylation of PKA-phosphorylated CREB and enhances CREB transcriptional regulation in rat liver nuclear extracts (266). This result is in contradiction with the results reported by Alberts *et al.* in which microinjection of a plasmid encoding simian virus 40 small t antigen had no effects on the phosphorylation state of CREB in stimulated or unstimulated NIH 3T3 cells (264). Until recently, the phosphorylation of KID was believed to allosterically change the conformation of the transactivation domain by bringing the glutamate-rich (Q-rich) regions flanking KID into an active conformation for positive interaction with the TATA box complex of factors involved in the initiation of transcription. However, the recent finding of a CREB-binding protein (CBP) which only binds phospho-CREB (when CREB is phosphorylated on Ser133) (271) could be the missing link between transcription factors which bind to their responsive elements far away from the TATA box and the basal transcription machinery. Phospho-CREB recruits CBP to bridge the distantly positioned CRE-CREB complex and TFIIB (272) which interact with the TATA-box-binding protein (TBP) and functions to recruit polymerase II holoenzyme (273). CBP also mediates the activation of mitogen responsive genes by interacting with c-Jun. This interaction, which augments c-Jun-directed transcription, only occurs when c-Jun is phosphorylated at Ser63 and Ser73. (274). Phosphorylation-dependent interactions between transcription factors and CBP suggest how phosphorylation of transcription factors is correlated with their activation consequences and also demonstrates how enhancers can stimulate promoters from a distance.

The activity of transcription factors other than CREB such as c-Jun, c-Myc and C/EBPβ is regulated by phosphorylation-induced transactivation. C-Myc activation occurs after its phosphorylation by MAP-ERK kinases (230, 275), and c-Jun is activated by a kinase which is stimulated in response to expression of activated Ha-Ras (233, 276, 277) and other transforming proteins (278). The phosphorylation sites responsible for these effects are located, like for CREB, in the transactivation domain of these proteins. In contrast, CaM-kinase II phosphorylation of C/EBPβ within its leucine zipper dimerization domain induces transactivation. This phosphorylation occurs on Ser276 and does not affect C/EBPβ-DNA binding activity.

C. *CaM-dependent protein kinases and phosphatase*

Ca^{2+}/CaM-dependent protein kinases

The calcium-calmodulin dependent protein kinase family can be divided into 2 groups. The kinases within the first group are myosin light chain kinase (MLCK), phosphorylase kinase and CaM-kinase III. Each of these kinases phosphorylates a unique and specific substrate and have mainly been studied in non-neuronal tissues. MLCK phosphorylates the regulatory light chain of myosin which is an important step in the initiation of smooth muscle contraction (113, 279-281). Phosphorylase kinase phosphorylates glycogen synthase and glycogen phosphorylase (282). CaM-kinase III participates in the regulation of protein synthesis by phosphorylating the elongation factor 2 (127, 126, 283). The second group of kinases is composed of CaM-kinases I, II and IV. These kinases, unlike the first group, have a wide substrate specificity. As the kinases of the first group do not seem to be relevant to transcription, I will focus this section on the second group of CaM-kinases that have a wider substrate specificity than the first one. The CaM-kinases composing this group are very similar in their subunit structure and have numerous substrates in common .

1.*CaM-kinase I*

CaM-kinase I, a monomeric enzyme, has been originally characterized in neuronal tissue on its ability to phosphorylate site 1 of synapsin I (284, 285), however it has a widespread cell and tissue distribution (125). To date, in addition to synapsin I and II (285) (286, 287), other proteins substrates of CaM-kinase I have been characterized *in vitro* such as smooth muscle myosin light chain (284), CREB (157) and the cystic fibrosis transmembrane conductor (CFTR) (288). CaM-kinase I phosphorylates synapsin I, CREB and CFTR at sites which are also phosphorylated by PKA. The site in synapsin I phosphorylated by CaM-kinase I is present in the following sequence: -Y-L-R-R-R-L-S*-D-S-N-F-. The peptide where the second Ser has been mutated to Ala is as good a substrate as

the original peptide (289). Studies on the phosphorylation of a number of peptide substrate analogs to this sequence demonstrated that the arginine residues at position P-2, -3, and -4 are important for full activity, but that the arginine at position -3 is the most critical (289). Thus, its widespread tissue distribution and the fact that other non-neuronal substrates have been identified suggest that CaM-kinase I may function as a ubiquitous multifunctional protein kinase.

Two forms of CaM-kinase I, Ia and Ib, have recently been purified from rat brain (290), with molecular weights of 43 and 39 kD respectively. CaM-kinase Ia autophosphorylated in a Ca^{2+}/CaM-dependent fashion exclusively on threonine residues, while CaM-kinase Ib autophosphorylation showed Ca^{2+}/CaM-independence and occured on both serine and threonine. Proteolytic digestion of autophosphorylated CaM-kinase Ia and Ib yielded phosphopeptides of different Mr (290). CaM-kinase Ib is also about 20-fold more sensitive to CaM than Ia. Preincubation of CaM-kinase Ia in the combined presence of Ca^{2+}/CaM and Mg^{2+}/ATP led to a time-dependent increase in its site 1 synapsin 1 peptide kinase activity of up to 15-fold. CaM-kinase Ib activity is dependent upon phosphorylation by a regulating kinase which is resolved from Ib during its purification (291). CaM-kinase Ia activator has recently been described; its estimated molecular weight on SDS-PAGE is 52 kD. In the absence of activator, CaM-kinase Ia activity was only 2% of its maximal activity after activation with the activator. In its activated state, CaM-kinase Ia remains dependent on Ca^{2+}/CaM. Activation is rapid and requires Ca^{2+}/CaM and Mg^{2+}/ATP. This activator is also capable of Ca^{2+}-dependent binding to CaM-Sepharose (291).

A CaM-kinase I cDNA has recently been cloned from both rat and bovine brain cDNA libraries (125); its calculated molecular weight is 37.5 kD (125). CaM-kinase I is encoded by a single gene and autophosphorylates on Thr-177 which is located at a position equivalent to that of the threonyl residue (Thr-197) autophosphorylated in PKA. Autophosphorylation of this residue does not seem to affect its Ca^{2+}/CaM-dependent activity (125). Recent studies (292) on recombinant truncation mutants of CaM-kinase I

suggest that the activity of the enzyme is regulated by an autoinhibitory domain (293) and that the kinase remains fully CaM-dependent after autophosphorylation.

2. *CaM-kinase II*

CaM-kinase II, also called multifunctional Ca^{2+}/CaM-dependent protein kinase II (8, 11, 294), has been purified from a wide variety of tissues and species (295-300). The proteins obtained exhibit properties that are characteristic of the CaM-kinase II isozyme family: large native molecular mass (300-700 kDa), subunits of 50 to 62 kDa as determined by SDS-PAGE, similar catalytic properties with a wide range of substrates and a very strong Ca^{2+}/CaM-dependent autophosphorylation which results in the partial loss of calcium-dependency. CaM-kinase II is most abundant in the brain, constituting approximately 0.3 % of total brain protein (301) and up to 2% of the total protein in the hippocampus. CaM-kinase II is particularly concentrated at the synapse where it can constitute up to 20-50% of the total protein in the postsynaptic density (302-305).

CaM-kinase II cDNAs for four different isoforms of the enzyme have been cloned and sequenced from rat brain libraries. Two brain-specific clones encode the α and β subunits (306-309). Alternative splicing generates both the β mRNA and a distinct β' isoform (307), while α generates α and α_B. The cDNA for the α isozyme encodes a protein of 54 kDa on a 5 kB message, while the cDNA for the β subunit translates to a protein of 60 kDa contained on a 4.8 kB message. α and β are 91% identical in amino acid sequence in the N-terminal half, and 76% identical in the C-terminal half (307). Two other isoforms, γ and δ, have been cloned from rat brain (310, 311), and each of these is a distinct gene product with approximately 85% homology in amino-acids and 75% homology in nucleotides to the α subunit. The γ and δ isoforms are present in essentially every tissue. The γ and δ isoforms have a molecular weight of 59 and 60 kDa, respectively, and generate by alternative splicing γ_A, γ_B, γ_C (312, 313) and at least six isoforms of δ (314, 315). Holoenzyme composition varies in different brain regions (316, 317) as well as during development (318).

Domain localization has been determined by analyses of the deduced amino-acid sequences (307, 309) : the N-terminal half of the subunit contains the highly conserved kinase motifs, the central portion comprises the regulatory elements, and the C-terminus is thought to be involved in subunit assembly and/or subcellular localization (8).

Studies on the regulation of CaM-kinase II have provided evidence for a model (9) in which CaM-kinase II is kept almost totally inactive in its basal state by the presence of an autoinhibitory domain (293). As proposed as early as 1978 by Jackie Corbin for the cAMP-kinase (319), some kinases (e.g., MLCK, PKC, CaM-kinases I, II and IV etc.) contain a sequence that closely resembles the consensus phosphorylation-site sequence in their respective protein substrates. This 'substrate-like' sequence within the kinase, or within the regulatory subunit of the kinase in the case of PKA, is predicted to interact with and inhibit the catalytic site of the enzyme and is called an autoinhibitory domain. Furthermore, if the substrate-like sequence present in the kinase does not contain a phosphorylatable amino-acid, the substrate-like sequence is called a pseudo-substrate. Such a sequence exists in CaM-kinase II. CaM-kinase II can be activated either by binding of Ca^{2+}/CaM or by autophosphorylation on Thr286 in the α subunit (Thr287 for the β subunit) which converts the kinase into a partially Ca^{2+}-independent species (8). This effect can be reversed by protein phosphatase 1 (320), 2A (321-324) and 2C (325). Autophosphorylation of CaM-kinase II on Thr286 is an intraholoenzyme mechanism which is intersubunit-catalyzed (326, 327). Another residue autophosphorylated and important for CaM-kinase II regulation is Thr306. Its autophosphorylation results in a partial loss of total kinase activity by blocking binding of CaM to the regulatory domain. Unlike the autophosphorylation of Thr286, autophosphorylation of Thr306 is *via* an intrasubunit-catalyzed mechanism (326).

The minimal consensus phosphorylation sequence was determined to be -R-X-X-S/T- (328-330). When the Arg residue was replaced by Ala in a synthetic peptide, this peptide was not significantly phosphorylated by CaM-kinase II, demonstrating the essential nature of an Arg three residues amino terminal (p-3 position) of the phosphorylated residue.

Addition of a second Arg in the p-2 position (-R-R-X-S/T-) results in a 10-fold decrease in the ratio of Vm/Km for CaM-kinase II whereas this substitution is a strong positive determinant for phosphorylation by PKA. Thus CaM-kinase II and PKA have some overlap in substrate specificity determinants.

In general, the sequence -R-X-X-S/T- has been found in most CaM-kinase II protein sustrates. However, a few exceptions have been reported (8) including the autophosphorylation sites Thr382 (-P-Q-T-T*-) (331) and Thr 305/306 (-A-I-L-T-T-) (332), phosphorylation sites in acetyl-CoA carboxylase (-G-S-V-S*-) (333), pyruvate kinase (-A-Q-L-T*-) (334) and the protein Vimentin (-S*-X-D-) (335). These results suggest that Arg is not the only determinant involved in substrate recognition. Secondary and tertiary conformations of substrate proteins can also influence their rates of phosphorylation.

As CaM-kinase II phosphorylates a wide range of proteins *in vitro* and *in situ* that are involved in neuronal functions (11), it is likely that these phosphorylation are regulating many different neuronal cellular processes. CaM-kinase II has been found to be essential in long-term potentiation (a model of neuronal plasticity) (336), in nuclear envelope breakdown (337) and in the release of glutamate and norepinephrine (338). CaM-kinase II is also believed to act as a postsynaptic sensor of synaptic activity (339-343). CaM-kinase II has also been shown to positively regulate calcium current in smooth muscles cells (344). Expression of a constitutive form of calcium/calmodulin-dependent protein kinase II leads to arrest of the cell cycle in G2 (345). CaM-kinase II has been shown to mediate inactivation of the M phase promoting factor (MPF) and cytostatic factor (CSF) upon fertilization of Xenopus eggs (346). The specific inhibition of CaM-kinase II prevents Ca^{2+} from triggering sister chromatid segregation in CSF extracts containing *in vitro*-assembled metaphase spindles (347). Finally, as described above, CaM-kinase II regulates transcription of the prolactin gene (169). Several laboratories have suggested that CaM-kinase II could positively regulate transcription of genes whose promoter contain a CRE site

in their promoter through phosphorylation of the transcription factor CREB (157, 164, 165), while others reported no such effects (169).

3. *CaM-kinase IV*

CaM-kinase IV, also called CaM-kinase GR because of its abundance in granullar cerebellar cells, is a recently cloned protein kinase of 53 kDa encoded by a single gene that has strong sequence homology with the catalytic and regulatory domains of CaM-kinase II (128) (129) (130). One of the major structural difference between CaM-kinase II and CaM-kinase IV is their C-terminus. The highly acidic nature of its C-terminus probably accounts for the monomeric structure of CaM-kinase IV. In addition to the cerebellum, CaM-kinase IV is also very abundant in the thymus and the testis (129, 348-350).

Our laboratory has recently demonstrated, using site-directed mutagenesis and synthetic inhibitory peptides, that CaM-kinase IV, like many other CaM-dependent kinases and phosphatases (293) does contain an autoinhibitory domain (434). The specific activity of purified CaM-kinase IV, even in the presence of Ca^{2+}/CaM, is about 10% of other kinases. The purified enzyme has been reported to be activated 10-fold upon autophosphorylation (351, 352), but the bacterially-or bacullovirus-expressed CaM-kinase IV is weakly activated by autophosphorylation (353, 354). A brain extract stimulates activation of the bacterially-expressed CaM-kinase IV (353), suggesting that the purified preparation from cerebellum was contaminated by another kinase. It has also been demonstrated that activation of T cell antigen receptor/CD3 complex in Jurkat cells produces a 17-fold increase in total CaM-kinase IV activity in the cell extract, indicating a physiological mechanism of stable activation (355). Our laboratory recently demonstrated that CaM-kinase IV is strongly activated through phosphorylation by a 68 kDa CaM-kinase IV kinase (434). The consensus phosphorylation sequence for CaM-kinase IV is not clear yet. The physiological substrates and function(s) of CaM-kinase IV also remain to be determined but its nuclear localization (356) renders it a very attractive candidate for Ca^{2+} regulation of gene transcription. Purified CaM-kinase IV phosphorylates numerous endogenous brain cytosol proteins (349)

and shares with CaM-kinase II *in vitro* substrates such as myelin basic protein, myosin light chain, tyrosine hydroxylase and tau protein. However casein and tubulin, which are good substrates for CaM-kinase II, are not phosphorylated by CaM-kinase IV, whereas histone H1 and Rap-1b (a Ras related GTP-binding protein) are good substrates for IV and not for II (349, 357). Synapsin I is phosphorylated on both sites I and II by CaM-kinase IV (at a ratio 1:2), in contrast to CaM-kinase II that only phosphorylates site II (348) and CaM-kinase I that only phosphorylates site I (284, 285). The study of the regulatory mechanisms of CaM-kinase IV activation are currently under investigation in our laboratory.

Ca^{2+}/CaM-dependent protein phosphatase,calcineurin

Calcineurin (CaN), the neuronal form of protein phosphatase 2B, is the only known Ca^{2+}/CaM-dependent Ser/Thr protein phosphatase. It is a major brain protein, accounting for 1% of total brain protein as opposed to 0.1 to 0.05% in other tissues (358, 359). Relative to protein phosphatases 1 and 2A, CaN has a narrow specificity (360) and is insensitive to the inhibitor okadaic acid (361, 362). Type 2B phosphatases are heterodimers composed of the catalytic A subunit (57-61 kDa) and a regulatory B subunit (19kDa) present in a 1:1 molar ratio (363).Three genes for the A subunit have been described, and alternative splicing of each generates additional isoforms (363). A single gene for the B subunit has been described (12).

The myristylated B subunit is an EF-hand Ca^{2+} binding protein with 35% homology with CaM (118, 359). However the two proteins are functionally distinct (12). The region of CaN A which binds CaN B is distinct from the CaM-binding domain (364). Ca^{2+} binding to CaN B does not affect the tight interaction between the two subunit, but it does cause conformational changes in both subunits and modestly activates basal CaN phosphatase activity. The A subunit contains a central CaM-binding domain (365) and a C-terminal autoinhibitory region (293, 366, 367). Limited proteolysis of CaN removes the region COOH-terminal of the CaM-binding domain and results in levels of Ca^{2+}-

independent activity similar to the Ca^{2+}/CaM-stimulated activity of the non-proteolyzed enzyme (368, 367). Expressed CaN A subunit has a very low phosphatase activity which is synergistically activated 200-fold by CaM plus the CaN B subunit (369). Kinetics studies have shown that Ca^{2+}/CaM activates CaN by increasing the Vmax whereas Ca^{2+}-binding to the B subunit decreases the Km, suggesting that the Ca^{2+}-induced conformational changes in the B and the A subunits increase the affinity of the catalytic domain for substrate (367). However the mechanism by which binding of the B subunit to the A subunit alters the phosphatase kinetic properties remains to be determined. Thus, the phosphatase activity of CaN is subject to a dual Ca^{2+} control mediated by two different Ca^{2+}-binding proteins; CaN B and CaM.

From the finding of numerous substrates of CaN *in vitro* (370) and the use of cell-permeable specific inhibitors (e.g.; FK506) of its activity, CaN has recently been shown to be involved in the regulation of several physiological functions such as long-term-depression (371), regulation of Na^{+} channels (372), M current K^{+} channels, NMDA receptors (373), neurotransmitter release (374), regulating the heat-stable inhibitors of PP1 (inhibitor-1 and DARRP-32) (359) and regulation of transcription (see above).

II

RESULTS

Roles of Calmodulin-Dependent Protein Kinases and Phosphatase in Calcium-Dependent Transcription of Immediate Early Genes

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ABSTRACT

Recent studies indicate multiple mechanisms are involved in Ca^{2+}-stimulation of gene expression. We have used cell-permeable, specific inhibitors of calmodulin-dependent protein kinases (CaM-kinases) and phosphatase (calcineurin) to investigate the involvement of these enzymes in transcriptional regulation of three immediate early genes (IEGs) in PC12 cells stimulated with A_{23187} or KCl. Preincubation of PC12 cells with the CaM-kinase inhibitor KN-62 blocked autophosphorylation of CaM-kinase II in response to stimulation by the Ca^{2+} ionophore A_{23187}. KN-62 treatment also resulted in a 60-70% inhibition of Ca^{2+}-dependent transcription of *c-fos*, NGFI-A (*zif/268*) and NGFI-B (*Nur/77*) as assessed by either Northern or nuclear run-on analyses. Preincubation with the calcineurin inhibitors FK-506 or cyclosporin A strongly enhanced expression of NGFI-A and blocked transcription of NGFI-B, but it had no significant effect on Ca^{2+}-stimulated transcription of *c-fos*. Both FK-506 and KN-62 were specific for Ca^{2+}-stimulated transcription as neither effected transcription in response to forskolin or phorbol ester (TPA) treatment.

This is the first report of CaM-kinase and calcineurin involvement in transcriptional regulation of NGFI-A and NGFI-B. Activation of CaM-kinases and calcineurin, in response to elevated intracellular Ca^{2+}, would exert antagonistic effects on transcription of NGFI-A. Since inhibition of either the kinase or phosphatase decreased transcription of NGFI-B by 60-90%, this suggests that each enzyme is necessary but not sufficient for Ca^{2+}-stimulation. These results indicate that CaM-kinases and calcineurin can mediate broad and complex regulation of Ca^{2+}-stimulated gene expression.

INTRODUCTION

In the nervous system synaptic transmission often regulates gene expression through second messenger pathways (375) Expression of immediate early genes (IEGs), which does not require new protein synthesis, is activated within minutes (16). Many of these IEGs encode DNA binding proteins which serve as transcription factors for the late response genes whose expression can modify long-term neuronal phenotype and/or excitability (110). Since many neuronal IEGs are responsive to elevations in intracellular calcium, the regulatory roles of Ca^{2+}/calmodulin-dependent protein kinases (CaM-kinases) and phosphatase, both of which are especially abundant in neuronal tissues, are of great interest.

Transcriptional regulation of IEGs, especially *c-fos*, has been extensively studied in PC12 cells. Enhanced expression of *c-fos* can be mediated by multiple signal transduction pathways involving cAMP, Ca^{2+} and protein kinase C (376) The best characterized system is the cAMP response which requires phosphorylation of the transcription factor CREB (249). CREB binds to the cAMP responsive element (CRE) (160) in the promoter region of numerous genes (161), and its phosphorylation on Ser^{133} by cAMP-kinase (PKA) enhances transcriptional initiation (163). CREB can also be phosphorylated on Ser^{133} by several members of the CaM-kinase family (157, 377), and this has been proposed as a mechanism to explain Ca^{2+}-dependent regulation of CRE-containing genes (157). For example, co-transfection of PC12 cells with a GAL4-CREB fusion construct confers Ca^{2+}-inducibility on a *c-fos* reporter gene containing a GAL4 binding site (157). Mutation of Ser^{133} to Ala reduces by 80 % both the cAMP- and Ca^{2+}-responsiveness. Furthermore, KN-62, a cell-permeable CaM-kinase inhibitor, blocks Ca^{2+}-induction of a transfected construct containing the glucagon CRE in front of a reporter gene (165). KN-62 inhibits many members of the CaM-kinase family (377, 378) competitively with Ca^{2+}/CaM, but it does not inhibit PKA or PKC (379). We have recently demonstrated that KN-62 inhibits

CaM-kinase IV (377) (also called CaM-kinase GR), a relatively new member of the CaM-kinase family (129, 348, 380) that has significant nuclear localization (356). We (377) and others (1, 381) have also demonstrated that CaM-kinase IV, but not CaM-kinase II, is capable of activating transcription through phosphorylation of CREB.

Although extensive evidence exists for a role of CaM-kinases in Ca^{2+}-dependent transcriptional regulation, there is evidence that additional pathways or interactions may exist. For example, inhibition of PKA appears to block Ca^{2+}-dependent induction of *c-fos* in PC12 cells (211). In hippocampal neurons both membrane depolarization and stimulation of NMDA receptors result in elevations of intracellular Ca^{2+}, activation of CaM-kinase II and induction of *c-fos* (158). However, induction of *c-fos* by membrane depolarization, but not by NMDA receptor stimulation, is blocked by KN-62. The NMDA response appears to be mediated by the serum response element (SRE), not the CRE (158). The SRE also appears to be involved in Ca^{2+}-induction of *c-fos* in response to treatment of fibroblasts with thapsigargin, an inhibitor of the Ca^{2+}/ATPase pump (198). Furthermore, in fibroblasts, macrophages and Myeloid leukemia cells, Ca^{2+} induction of *c-fos* may largely involve relief of a block in transcriptional elongation in the first intron rather than transcriptional initiation (199-201). Lastly, Ca^{2+}-stimulation of interleukin-2 expression in T cells involves the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin (CaN) (190, 191). Dephosphorylation of transcription factor NF-AT by calcineurin is required for Ca^{2+}-induction of interleukin-2 (194). Cyclosporin A and FK506, which are potent and specific calcineurin inhibitors (185), block Ca^{2+}-stimulated interleukin-2 transcription (195).

Since it is clear that multiple mechanisms can be involved in Ca^{2+}-induction of numerous genes, we have investigated the roles of CaM-kinases and calcineurin in the regulation of three immediate early genes, *c-fos*, NGFI-A (also called *zif/268*) and NGFI-B (*Nur/77*), which are expressed in PC12 cells. NGFI-A codes for a member of the zinc finger transcriptional activator family (28, 77), and NGFI-B codes for a member of the

steroid receptor super family of transcription factors (32, 33). Using cell-permeable inhibitors of CaM-kinases and calcineurin, we demonstrate that CaM-kinases are involved in the Ca^{2+}-induction of all three IEGs, suggesting a broad role for CaM-kinases in transcriptional regulation. Our results also show that calcineurin can mediate either a synergistic or antagonistic response to Ca^{2+}-dependent transcriptional regulation. This is the first report of involvement of CaM-kinases and calcineurin in transcriptional regulation of NGFI-A and NGFI-B.

EXPERIMENTAL PROCEDURES

Cell culture

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal calf serum (FCS) and 5 % horse serum (HS) on Primaria dishes. Cell were maintained in a 5% CO_2 incubator at 37°C. Prior to treatments, the cells were incubated in serum-free medium for 12 hours.

^{32}P-cell labeling and immunoprecipitation

Cells were plated at a density of 1.4×10^6 cells per 35mm dish for 2 days. The cells were incubated with [^{32}P]orthophosphate (0.45 mCi) in 1.5 ml of phosphate free DMEM for 1 hour prior to initiation of various treatments. After treatment, the medium was quickly aspirated, and the cells were frozen in liquid nitrogen. The cells were thawed and scraped with 1 ml of RIPA buffer (60 mM Tris-HCl pH 7.5, 180 mM NaCl, 6 mM EDTA, 18 mM sodium pyrophosphate, 60 mM NaF, 1.2 % NP-40, 1.0 mM PMSF, 6 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin A, 0.1 % SDS, 1 µM microcystin-LR), the lysates were centrifuged and the supernatants collected for immunoprecipitation. 5 µl of the samples were TCA (trichloroacetic acid) precipitated, dissolved in 0.1 N NaOH, and reprecipitated with TCA. Total radioactivity was equated for the samples before the immunoprecipitation. The supernatants were then transferred to new tubes and incubated with specific antiserum for CaM-kinase II, for 16 hr at 4 °C. Protein-A-Sepharose was added for 2 hrs, and the PAS-antibody-antigen complexes were pelleted and washed four times with 50 mM Tris-HCl (pH 8.3), 0.6 M NaCl, 0.5 % Triton X-100 plus 0.1 mM PMSF. Immunoprecipitates were solubilized in SDS sample buffer followed by boiling for 2 min. Protein were fractionated by SDS-PAGE (382), and phosphoproteins were visualized by autoradiography.

Northern analyses

Cells were plated at a density of 4 X 10^6 cells per 100 mm dish 2 days prior to treatment as indicated in the figures legends. Total RNA was isolated according to Cathala *et al.*. (383). Aliquots of 20 µg total RNA were subjected to electrophoresis in 1.4 % agarose gels containing 0.66 M formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA and blotted to Hybond membranes. The blots were baked for 2 hours at 80 °C, and hybridizations were performed at 42-45 °C for 12-18 hours in 50 % de-ionized formamide, 5 % SDS, 0.4 M sodium phosphate, pH 7.2, 1 mM EDTA, 1 mg/ml BSA using ^{32}P-labeled DNA probes. After washing and drying, the blots were subjected to autoradiography. The cDNA probes were as follows: for *c-fos* a 1027 bp Ava I fragment was kindly provided by Dr. T. Curran (Roche Institute of Molecular Biology); for NGFI-A, a 1.7 kb cDNA was provided by Dr. James Douglass (Vollum Institute); for NGFI-B 2.5 kb cDNA was provided by Dr J. Milbrandt (Washington University).

Nuclear run-on analyses

PC12 cells were lysed in a hypotonic buffer containing 10 mM Tris, pH 7.4, 25 mM sodium chloride, 5 mM magnesium chloride and 0.05 % NP-40, and nuclei were isolated by centrifugation as described (384). The nuclei were collected in a buffer containing 20 mM Tris pH 9, 75 mM sodium chloride, 0.5 mM EDTA, 8.5 mM DTT and 50 % glycerol, and an aliquot was incubated with ^{32}P-CTP (385). The newly synthesized RNA was separated from free nucleotides by ethanol precipitation, and specific message was detected by hybridization of the RNA with nitrocellulose membranes on which cDNAs for each gene had been immobilized. The filters were then washed and subjected to autoradiography.

Other methods and materials

The PKA activation state was measured in PC12 cells as described (386, 387). T-test statistical analyzes were performed using the T-EASE program. The ^{32}P incorporation was quantified by densitometric scanning (Hoefer GS300-transmittance-reflectance scanning densitometer). [^{32}P]orthophosphate was purchased from ICN; [γ–^{32}P]ATP was from New England Nuclear; A_{23187}, forskolin, IBMX and TPA from Sigma Chem. Co.; KN-62 from Seigakagu; DMEM was from GIBCO and Primaria dishes from Falcon.

RESULTS

Involvement of CaM-kinases in calcium-dependent expression of immediate early genes.

Our strategy was to test the involvement of CaM-kinases in the Ca^{2+}-dependent transcriptional regulation of the three IEGs *c-fos*, NGFI-A and NGFI-B by use of the cell-permeable CaM-kinase inhibitor KN-62. We therefore wanted to confirm that KN-62 inhibited CaM-kinases in our PC12 cell preparation, and we chose CaM-kinase II as it had been previously demonstrated to be inhibited by KN-62 in PC12 cells (379). PC12 cells were metabolically labeled with $^{32}PO_4$, and treatment with 1 or 10 μM divalent cation ionophore A_{23187} increased by 2- to 3-fold ($p<0.01$, $n=3$) the ^{32}P-labeling of the immunoprecipitated CaM-kinase II compared to non-stimulated cells (Fig. 1). Preincubation of the cells for 30 min with 10 μM KN-62 before addition of A_{23187} inhibited ($p<0.05$, $n=3$) the enhanced autophosphorylation of CaM-kinase II (Fig. 1). Similar results were obtained when the cells were stimulated with 55 mM KCl (data not shown) rather than A_{23187}. These results confirmed that KN-62 was inhibiting CaM-kinases in PC12 cells under our incubation conditions.

We next tested the ability of KN-62 to inhibit Ca^{2+}-stimulated mRNA levels. Fig. 2 illustrates a Northern blot of IEG expression at different times of stimulation with 5μM A_{23187} without or with preincubation with 10μM KN-62. As previously reported (16), *c-fos*, NGFI-A and NGFI-B mRNAs exhibited strong Ca^{2+}-dependent elevations, and the mRNA levels for the three IEGs peaked 30-60 min after stimulation by 5μM A_{23187}. When Ca^{2+}-free medium containing 0.1 mM EGTA was used, no induction of the IEGs by A_{23187} was observed (data not shown). Preincubation of the cells for 60 min with KN-62 prior to addition of A_{23187} resulted in a strong reduction in mRNA levels. Densitometric scans from eight different experiments were analyzed, and KN-62 gave a 60-70% inhibition of all three IEGs after 45 min of A_{23187} stimulation (Fig. 2, $p<0.0001$). Neither A_{23187} or KN-62 affected the mRNA levels for cyclophilin, a constitutively-expressed

gene that is commonly used as a control (388, 389). Similar inhibition by KN-62 was obtained when the intracellular concentration of calcium was raised by membrane depolarization with 55 mM KCl (data not shown).

Transcription of these three IEGs is also regulated by PKA and PKC (376), and some effects of Ca^{2+} are blocked by PKA inhibitors (211), suggesting that Ca^{2+} might stimulate CaM-dependent adenylate cyclase. To determine if A_{23187} was acting through activation of PKA, we determined the activation state of PKA in our experiments. A_{23187} had no effect on PKA whereas forskolin strongly increased its activation state (Fig. 3). Furthermore, KN-62 should have no effect on the PKA or PKC second messengers pathways if it is specifically inhibiting CaM-kinases. This was tested by stimulating the PC12 cells with forskolin to elevate cAMP and activate PKA or with TPA to activate PKC. As shown in Fig. 4A, KN-62 had no effect on the forskolin (10μM) or TPA (100 ng/ml) induction of the three IEGs, confirming the specificity of KN-62 for the CaM-kinase pathway.

To establish whether the effects of A_{23187} and KN-62 on mRNA levels reflect transcriptional regulation, nuclear run-on experiments were performed. Fig. 4B shows a representative experiment of the induction of the IEGs by a 15 min treatment with A_{23187} without and with KN-62 preincubation. The inductions of *c-fos*, NGFI-A and NGFI-B were similar to those previously reported in response to membrane depolarization with KCl (16). Again, KN-62 gave 80-90% inhibition for all three IEGs (Fig. 4B), demonstrating a strong inhibitory effect on Ca^{2+}-stimulation at the transcriptional level.

Involvement of calcium/CaM-dependent protein phosphatase in the calcium regulation of IEGs.

Since elevation of intracellular Ca^{2+} would be expected to also activate calcineurin, the Ca^{2+}/CaM-dependent protein phosphatase (370, 390), we wanted to ascertain if calcineurin played a regulatory role in IEG mRNA levels. Involvement of calcineurin was

determined by preincubation of the PC12 cells with FK506 or cyclosporin A, specific and potent inhibitors of this phosphatase (185). Fig. 5A shows a Northern blot of the three IEG mRNAs after A_{23187} stimulation without or with preincubation in the presence of 0.1 μM FK506 or 1 μM cyclosporin A (CsA) for 30 and 60 min, respectively. CsA and FK506, neither of which had any effect in the absence of A_{23187}, gave an 8-fold potentiation of the Ca^{2+}-stimulation of NGFI-A ($p<0.0001$, n=3) but blocked the Ca^{2+}-induction of NGFI-B ($p<0.0001$, n=3). No significant effect was observed on *c-fos* expression. FK-506 had a similar effect on the Ca^{2+}-responsiveness of the three IEGs when subjected to nuclear run-on analyses (Fig. 5B). These highly specific effects of calcineurin inhibitors were only observed for Ca^{2+}-induction of IEGs as FK506 preincubation was without effect when cells were stimulated with forskolin or TPA (Fig. 6).

The regulatory effects of calcineurin could be either direct or mediated indirectly through dephosphorylation of inhibitor 1 (I-1). Phosphorylated I-1 inhibits protein phosphatase 1 (PP1), so calcineurin can activate PP1 by dephosphorylating I-1 (391). PP1 is a general protein phosphatase that can dephosphorylate transcriptional factors including CREB (263). Thus, inhibition of calcineurin by FK506 could inhibit PP1 by preventing normal dephosphorylation of I-1. To determine if the calcineurin effect was being mediated by PP1, we preincubated cells with okadaic acid (OA), a potent inhibitor of protein phosphatases 1 and 2A. Okadeic acid (OA) did not mimic the pattern obtained with FK506 since the calcium-stimulated mRNAs for all three IEGs were inhibited by 1-100 nM OA or enhanced by 300-500 nM OA (Fig. 7). Neither FK506 (Fig. 5A) nor OA (Fig. 7) had significant effects by themselves at the concentrations used in this study. Thus, it is clear that FK506 and OA can have very different regulatory effects on Ca^{2+}-stimulation of NGFI-B.

DISCUSSION

Over the past five years mechanisms involved in Ca^{2+}/CaM-stimulated gene expression have begun to emerge. There is good evidence that the multifunctional CaM-kinase II is involved in transcriptional regulation through phosphorylation of C/EBPβ (174). It is also well established that the Ca^{2+}/CaM-dependent protein phosphatase calcineurin regulates interleukin-2 (IL-2) gene transcription through dephosphorylation of NF-AT (194). The present paper examines in PC12 cells the roles of CaM-kinases and calcineurin in transcriptional regulation of the three IEGs *c-fos*, NGFI-A and NGFI-B using specific inhibitors of these Ca^{2+}/CaM-dependent enzymes. Our results extend the regulatory involvement of CaM-kinases from *c-fos* (157) and prolactin (169) to also include NGFI-A and NGFI-B. We also demonstrate negative and positive roles for calcineurin in the transcriptional regulation of NGFI-A and NGFI-B, respectively.

The CaM-kinase inhibitor KN-62 blocked Ca^{2+}-dependent autophosphorylation of CaM-kinase II (Fig. 1) and reduced by 60-70% the Ca^{2+}-stimulated transcription of all three IEGs in response to the divalent cation ionophore A_{23187} as determined by both Northern (Figs. 2 and 4A) and nuclear run-on (Fig. 4B) analyses. This Ca^{2+} effect was not mediated by PKA through activation of Ca^{2+}/CaM-dependent adenylate cyclase as treatment of PC12 cells with A_{23187} had no effect on the activation state of PKA (Fig.3). The inhibitory effect of KN-62 we observed was specific for the Ca^{2+} response as preincubation with KN-62 had no effect on transcription of these IEGs in response to forskolin or TPA which stimulate the PKA or PKC pathways, respectively (Fig. 4A). This result is consistent with the known *in vitro* specificity of KN-62 which inhibits CaM-kinase II competitively with Ca^{2+}/CaM, and it therefore has no effect on PKA or PKC (379). In smooth muscle cells KN-62 can inhibit CaM-kinase II without inhibiting another Ca^{2+}/CaM-dependent protein kinase, myosin light chain kinase or altering intracellular Ca^{2+} responses to calcium ionophore as determined by Fura-2 measurements (392).

However KN-62 is not specific for CaM-kinase II as it does inhibit other members of the CaM-kinase family (378) including CaM-kinase IV (377).

Our results do not rule out a possible involvement of CaM-kinase(s) in the Ca^{2+}-dependent relief of an elongation block as described in non-neuronal cells (199-201) since our probes for the nuclear run-on experiments represented the entire cDNA sequence of the three genes. However, our results are consistent with the report of Sheng *et al.* (157) which suggested a role for a CaM-kinase in Ca^{2+}-stimulated transcription of *c-fos* through a calcium response element (CaRE) that binds CREB. The mechanism(s) by which CaM-kinase(s) enhances transcription of NGFI-A and NGFI-B is not known. Yoon *et al.* (73) identified a portion of the NGFI-B promoter essential for Ca^{2+} stimulation. Although this sequence does not contain a CRE/CaRE motif, there are two AP-1 like elements, one GC-rich sequence that includes the binding site for SP1 and a family of zinc finger proteins including the NGFI-A protein. The AP-1 like sequences in NGFI-B are very similar to the FAP element in the *c-fos* promoter which can act as a functional CRE in PC12 cells (202). Thus, it is possible that a CaM-kinase could be regulating transcription of these IEGs through phosphorylation of CREB, and we (377) and others (1, 381) have recently determined that transfected CaM-kinase IV can mediate transcriptional activation through phosphorylation of Ser^{133} in CREB. Although CaM-kinase II is an excellent catalyst for *in vitro* phosphorylation of CREB, CaM-kinase II phosphorylates equally a second site in additon to Ser^{133} (377). This second phosphorylation site is a strong negative determinant for transcriptional activation (1). Of course, Ca^{2+}-dependent transcriptional regulation of these IEGs through phosphorylation of transcription factors other than CREB cannot be excluded, and it has been demonstrated that Ca^{2+} induction of *c-fos* by thapsigargin in fibroblasts (198) and by glutamate in hippocampal neurons (158) is mediated by the serum response element (SRE) rather than the CRE. CaM-kinase IV can also phosphorylate the serum response factor (354), and it might therefore mediate transcriptional activation through the SRE. Since CaM-kinase IV has been identified in the nucleus (356) and it can

phosphorylate both CREB (377) and the SRF, CaM-kinase IV is an attractive candidate to mediate Ca^{2+}-dependent transcriptional regulation. Although it has been reported that CaM-kinase IV is not present in PC12 cells (349), it could be present in the nucleus at levels below the sensitivity of the detection method.

Our results also demonstrate that calcineurin is involved in transcriptional regulation of several genes in addition to interleukin-2. Cytosolic dephosphorylation of the transcriptional activator NF-AT by calcineurin appears to be necessary for translocation of NF-AT to the nucleus where it stimulates IL-2 transcription (393). This mechanism is involved in the action of immunosuppressants such as cyclosporin A (CsA) and FK506 which interact with cyclophilin and FK-binding protein, respectively. Each of these complexes specifically bind and inhibit calcineurin (185), thereby blocking T cell activation (195). Depending on the immune system cell line, CsA has been reported to either inhibit (394) or have no effect (179) on *c-fos* expression. In a pancreatic islet cell line CsA and FK506 inhibit CRE-mediated gene transcription after membrane depolarization (196). We have shown that CaN can efficiently dephosphorylate Ser^{133} in CREB *in vitro* (377), but we found no significant inhibitory effect of CsA and FK506 on *c-fos* expression in PC12 cells (Figs. 5A and 6). This may be a consequence of the fact that our experiments examined induction of the endogenous gene in the context of the whole promoter which also contains a Ca^{2+}-responsive SRE element (158, 381). Different mechanisms of induction of *c-fos* by calcium have been reported, and multiple enzymes such as CaM-kinases, CaN or MAP-kinase seems to be involved in these effects (158, 196). This multiplicity of Ca^{2+}-regulation could also explain why we never attained a total inhibition of calcium induction with either KN-62 or FK506. The calcineurin inhibitors had dramatic and opposite effects on Ca^{2+}-stimulation of NGFI-A and NGFI-B (Figs. 5A, 5B), but they were without effect on transcriptional regulation of these IEGs by forskolin and TPA (Fig. 6). This is the first report that calcineurin may be involved in transcriptional regulation of IEGs outside of the immune system. The locus of action of calcineurin is not

clear, but calcineurin has been detected in the nucleus of neurons (143), suggesting a potential nuclear mechanism. General regulatory effects of protein phosphatases 1 and 2A on all three IEGs are suggested by the effects of increasing concentrations of okadaic acid (Fig.7). Lower concentration (10 to 100 nM) of okadaic acid, which may predominantly inhibit phosphatase 2A, attenuated the Ca^{2+}-dependent transcription of all three IEGs. Higher concentrations (300-500 nM) enhanced transcriptional activation. In contrast to calcineurin which has a rather limited substrate specificity and exhibited very different effects on each of the three IEGs, phosphatases 1 and 2A are multifunctional and could be regulating multiple or general transcription factors. Since inhibition of phosphatase 1 by okadaic acid (Fig. 7) does not mimic the complex pattern of transcriptional regulation seen using FK506 (Figs. 5-6), this indicates that regulatory effects of inhibiting calcineurin by FK506 cannot be explained by preventing dephosphorylation by calcineurin of phosphatase inhibitor 1 with resulting inhibition of phosphatase 1.

In summary, our results extend our understanding of the involvement of CaM-kinases and calcineurin beyond *c-fos* and IL-2, respectively, to include interrelated transcriptional regulation of NGFI-A and NGFI-B. Elevation of intracellular Ca^{2+} in PC12 cells can activate CaM-kinases (168) and calcineurin (390). Activation of CaM-kinase would stimulate transcription of NGFI-A whereas calcineurin activation would attenuate NGFI-A transcription. Ca^{2+}-dependent transcriptional regulation of NGFI-B, on the other hand, is stimulated by activation of both CaM-kinase and calcineurin. Since inhibition of either enzyme decreases transcription of NGFI-B by 60-80%, this suggest that each enzyme is necessary but not sufficient for Ca^{2+} stimulation. It will now be important to identify the promoter elements and transcription factors regulated by these enzymes.

Acknowledgement: We would like to thank Drs. Hideyuki Yamamoto and Richard Goodman for helpful discussions and suggestions during the course of this project.

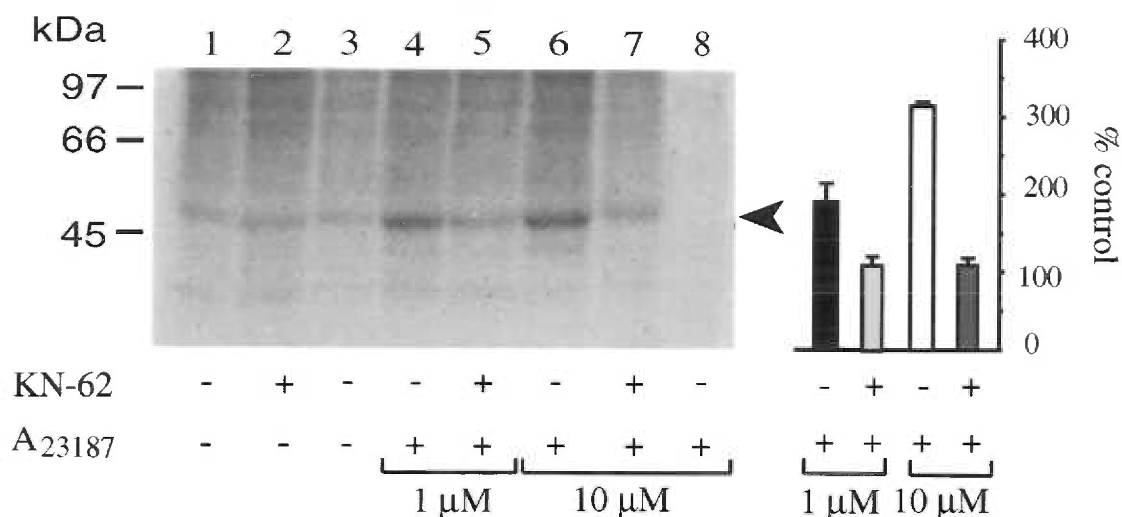

FIGURE 1: Effect of calcium ionophore on CaM-kinase II autophosphorylation in PC12 cells. PC12 cells were metabolically labeled with 32PO4 and preincubated with 0.1 % DMSO without (lanes 1, 3, 4, 6 and 8) or with (lanes 2, 5 and 7) 10 µM KN-62 for 30 min prior to treatment with another 0.1 % DMSO minus (lanes 1-3) or plus A23187 at 1µM (lane 4 and 5) or 10 µM (lanes 6, 7 and 8) for 5 min. Cells were solubilized and immunoprecipitated with antibody to CaM-kinase II (lanes 1 to 7) or preimmune serum (lane 8) as described in "Experimental Procedures". Immunoprecipitated proteins were separated by SDS-PAGE and subjected to autoradiography. The arrow marks the position of CaM-kinase II. 32P-CaM-kinase II was quantitated relative to non-stimulated (-A23187) controls from three separate experiments as shown in the bars to the right.

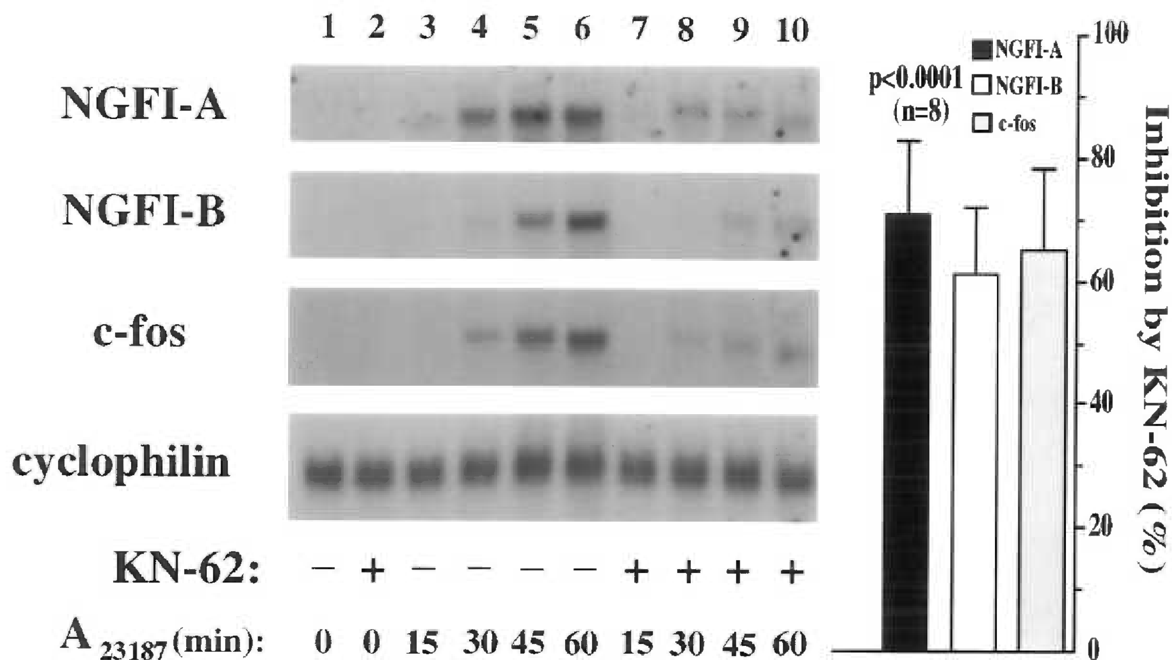

FIGURE 2: Temporal effects of calcium and KN-62 on IEG mRNA levels. PC12 cells were preincubated for 60 min with 0.1 % DMSO without (lanes 1 and 3-6) or with 10 μM KN-62 (lanes 2 and 7-10) before treatment with 0.1 % DMSO (lane 1 and 2, 60 min) plus 5 μM A23187 (lane 3-10) for the times indicated. Total RNA extraction and Northern blot analyses were performed as described in "Experimental Procedures". In the bars to the right the percent inhibition by KN-62 after 45 min stimulation with A23187 was quantitated for eight experiments.

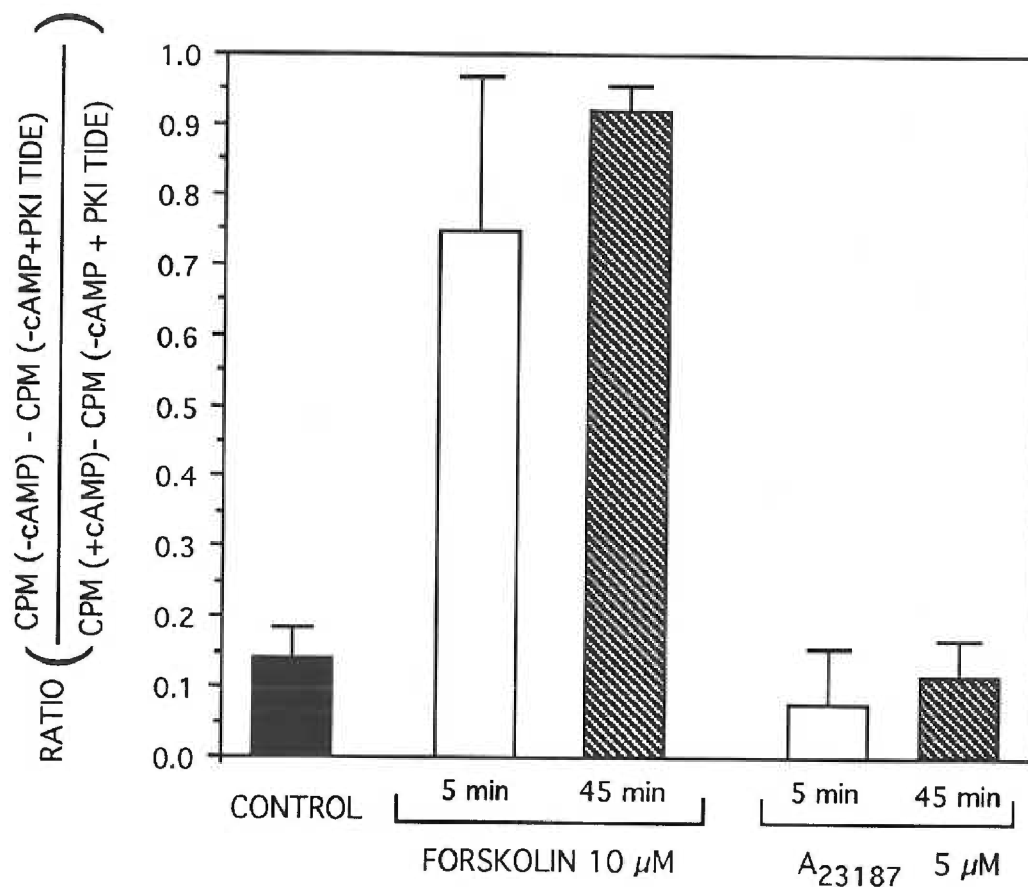

FIGURE 3: Effects of A23187 and forskolin on activation of PKA. PC12 cells were incubated with 5 mM A23187 or 10 mM forskolin for 5 or 45 min. The cells were centrifuged (14000 rpm for 15min) and the supernatant aspirated. The cells were lysed in a buffer containing 10 mM potassium phosphate, pH 6.8, 10 mM EDTA and 0.5 mM IBMX. The activation state (i.e., fraction of total kinase present as the active catalytic subunit) of PKA was determined using 60µM kemptide as substrate.

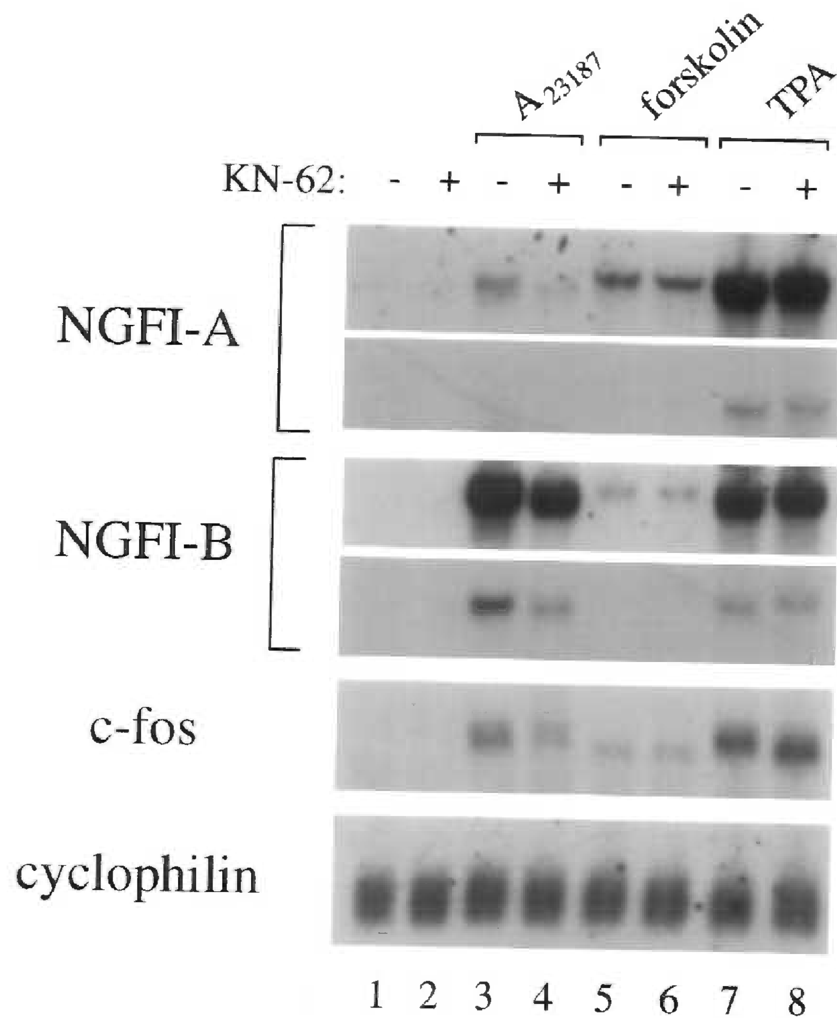

FIGURE 4A: Specificity of KN-62 for calcium stimulation of IEGs. PC12 cells were preincubated for 60 min with 0.1 % DMSO (-) plus 10 µM KN-62 (+) and stimulated for 45 min with 0.1 % DMSO (lanes 1 and 2) plus 5 µM A23187 (lanes 3 and 4), 10 µM forskolin (lanes 5 and 6) or 100 ng/ml of TPA (lanes 7 and 8). Total RNA extraction and Northern blot analyses were performed as described in "Experimental Procedures". For NGFI-A and NGFI-B short and longer exposures of the blots are shown.

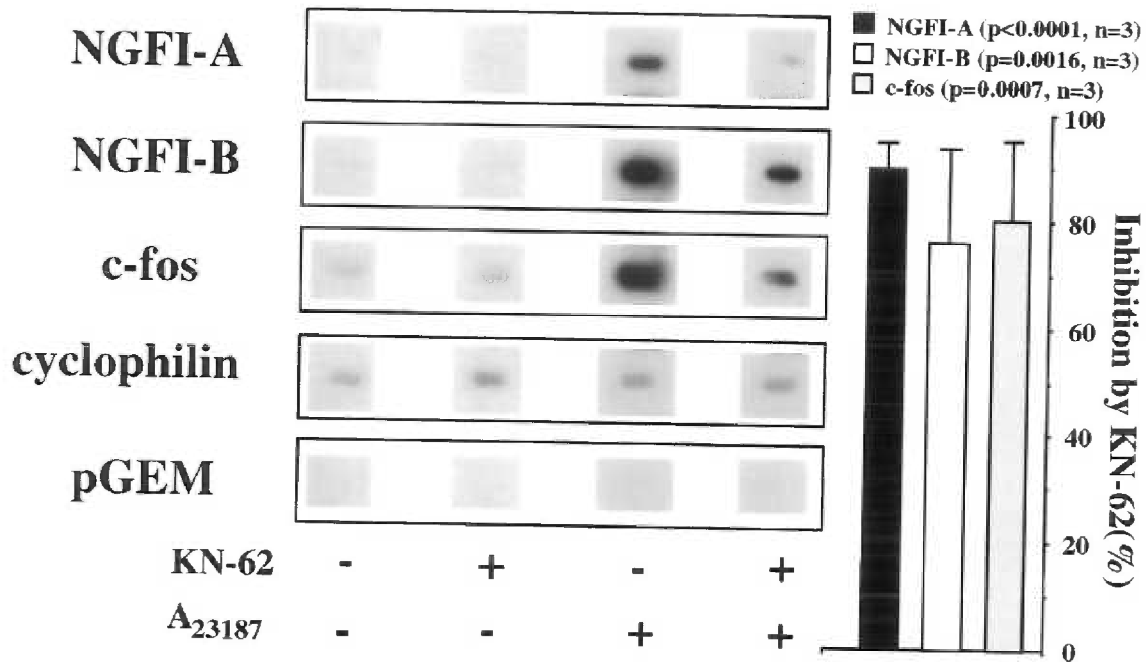

FIGURE 4B: Nuclear run-on analyses of KN-62 inhibitory effects. PC12 cells were preincubated for 60 min with 0.1% DMSO (-) plus 10 μM KN-62 (+) and stimulated with 5 μM A23187 in 0.1 % DMSO for 15 min. Nuclear run-on assays were performed as described in "Experimental Procedures". The percent inhibition of the A23187 response by KN-62 is quantitated at the right.

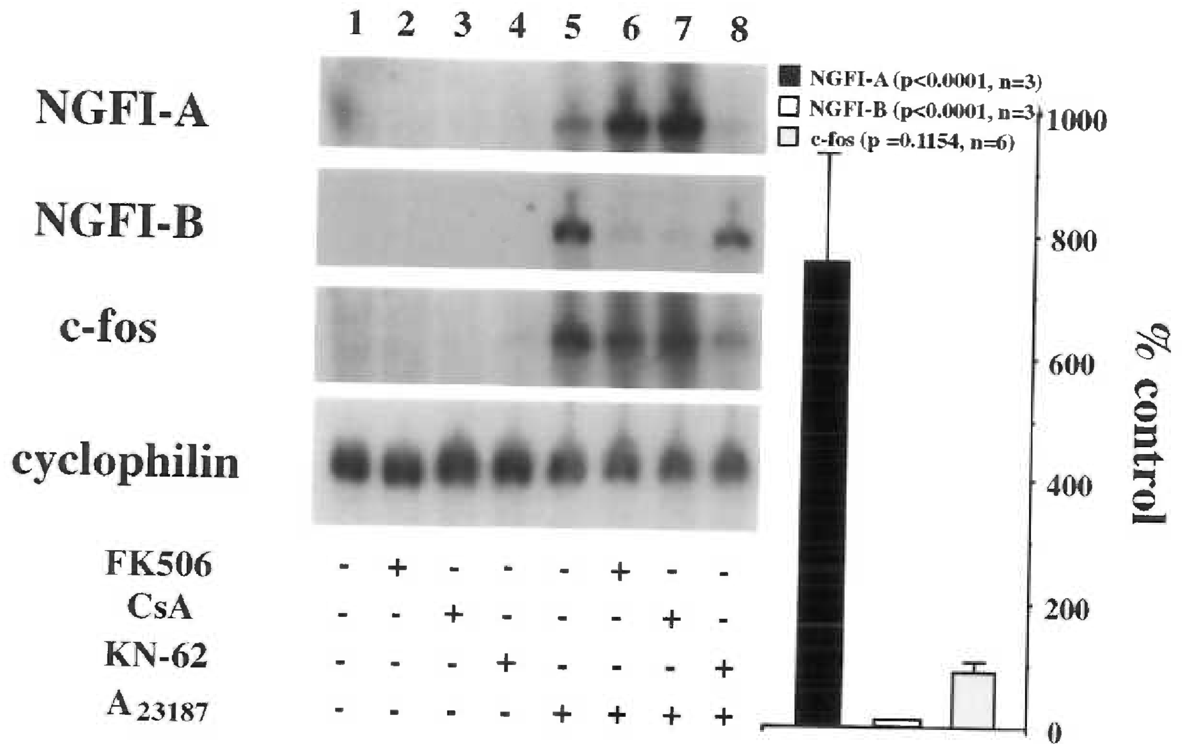

FIGURE 5A: Effects of CsA and FK506 on induction of IEGs. Northern analysis. PC12 cells were preincubated in 0.2 % methanol with the indicated combinations of 1μM CsA or 10 μM KN-62 for 60 min or 0.1 μM FK506 for 30 min before treatment for 45 min with 0.1 % DMSO (lanes 1-4) or 5μM A23187 (lanes 5-8). Total RNA extraction and Northern blot analyses were performed as described in "Experimental Procedures". The effects of FK-506 on A23187 stimulation are quantitated in the bars to the right.

A23187 forskolin TPA

FK506: - + - + - + - +

NGFI-A

NGFI-B

c-fos

cyclophilin

1 2 3 4 5 6 7 8

FIGURE 5B: Specificity of FK-506. Northern blot. PC12 cells were preincubated for 30 min with 0.2 % methanol (-) plus 0.1 µM FK506 (+) and stimulated for 45 min with 0.1 % DMSO (lanes 1 and 2) plus 5 µM A23187 (lanes 3-4), 10 µM forskolin (lanes 5-6) or 100 ng/ml of TPA (lanes 7-8).Total RNA extraction and Northern blot analyses were performed as described in "Experimental Procedures".

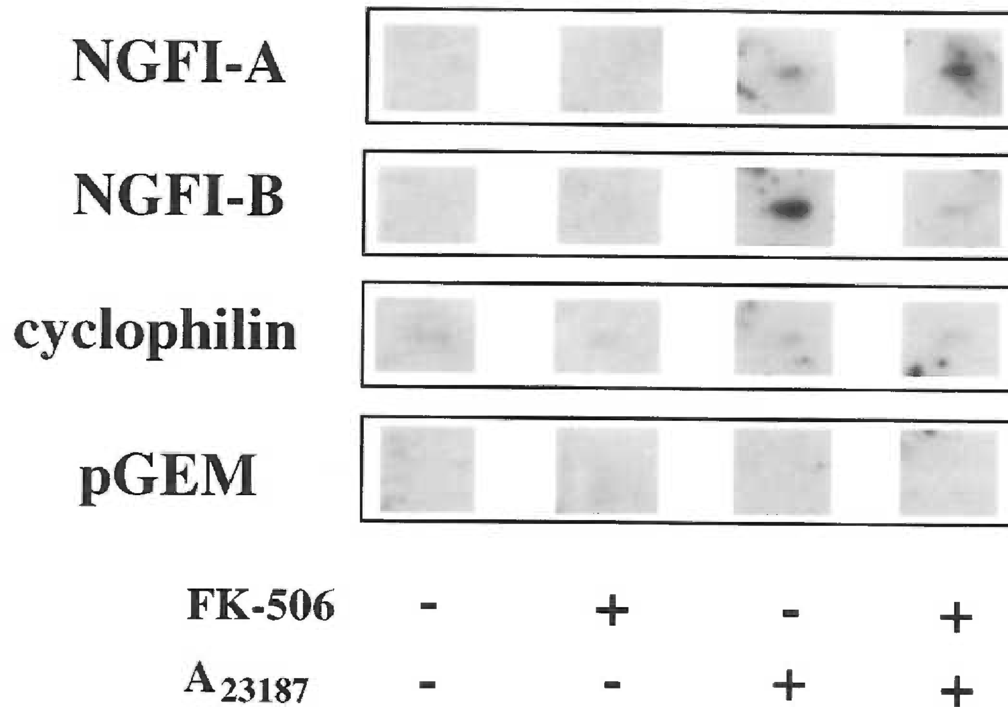

FIGURE 6: Effects of CsA and FK506 on induction of IEGs. Nuclear run-on analysis. PC12 cells were preincubated for 30 min with 0.2 % methanol (-) plus 0.1 μM FK506 (+) and stimulated with 5μM A23187 for 30 min. Nuclear run-on assays were performed as described in "Experimental Procedures".

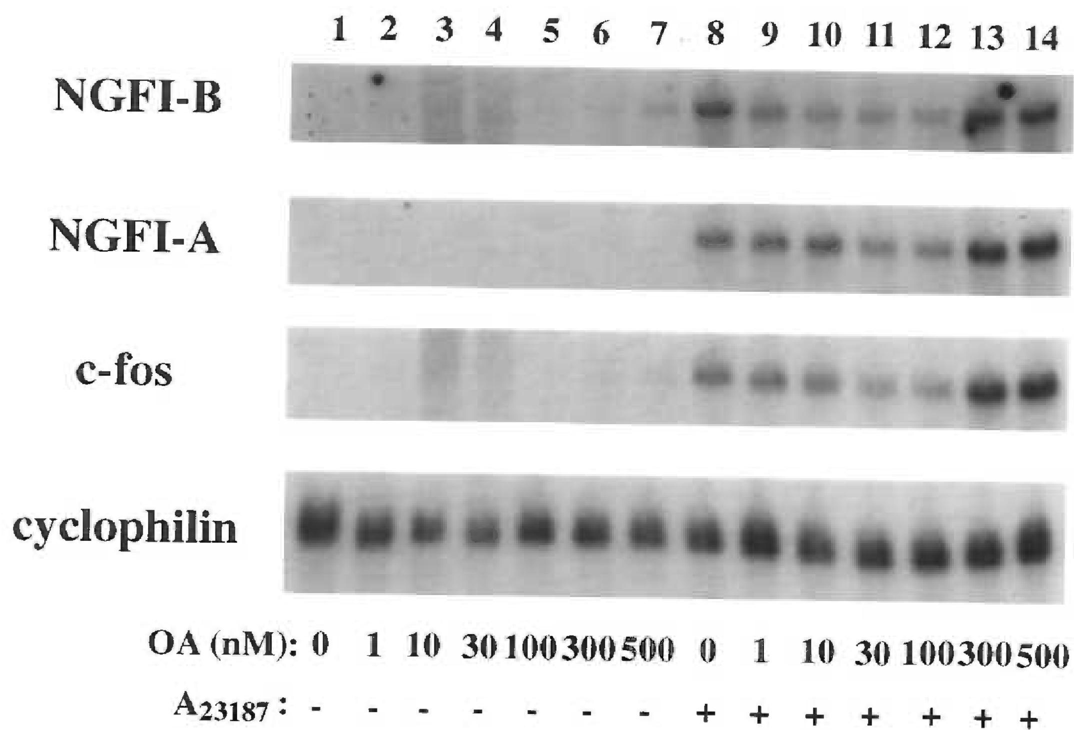

FIGURE 7: Effect of okadaic acid on IEGs induction by A23187. PC12 cells were preincubated for 60 min with 0.1 % DMSO (lanes 1 and 8) plus okadaic acid (OA) at the indicated concentration before treatment with 0.1 % DMSO (lanes 1-7) plus 5 μM A23187 (lanes 8-14) for 45 min. Total RNA extraction and Northern blot analyses were performed as described in "Experimental Procedures".

III

RESULTS

Characterization of Calcium/Calmodulin-Dependent Protein Kinase IV: Role in Transcriptional Regulation

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ABSTRACT

We have characterized Ca^{2+}/calmodulin-dependent protein kinase IV (CaM-kinase IV), expressed using the baculovirus/Sf9 cell system, to assess its potential role in Ca^{2+}-dependent transcriptional regulation. CaM-kinase IV was strongly inhibited *in vitro* by KN-62, a specific CaM-kinase inhibitor which suppresses Ca^{2+}-dependent transcription of several genes, so we tested whether CaM-kinase IV could stimulate transcription. Co-transfection of COS-1 cells by cDNA for CaM-kinase IV gave a three-fold stimulation of a reporter gene expression whereas co-transfection with CaM-kinase II gave no transcriptional stimulation. Since this transcriptional response was mediated by phosphorylation of CREB, we determined the kinetics and site-specificities of CaM-kinases IV and II for phosphorylating CREB *in vitro*. CaM-kinases IV and II and cAMP-kinase (PKA) all had similar K_m values for CREB (1-5μM), but the V_{max} of CaM-kinase IV was 40-fold lower than those of CaM-kinase II and PKA. Although all three kinases phosphorylated Ser^{133} in CREB, CaM-kinase II also gave equal phosphorylation of a second site which was not Ser^{98}. The two CREB phosphorylation sites were separately ^{32}P-labeled, and the abilities of protein phosphatases 1, 2A and 2B (calcineurin) to dephosphorylate them were tested. Our results show that all three phosphatases could dephosphorylate both sites, and calcineurin was a stronger catalyst for dephosphorylating site 1 (Ser^{133}) than for site 2.

These results indicate that CaM-kinase IV may be important in Ca^{2+}-dependent transcriptional regulation through phosphorylation of Ser^{133} in CREB. The fact that CaM-kinase II phosphorylates another site in addition to Ser^{133} in CREB raises the possibility that this second phosphorylation site may account for the suppressed ability of CaM-kinase II to enhance transcription through the CRE/CREB system. In addition multiple protein phosphatases, including calcineurin, may exert a modulatory effect on transcription depending on which site they dephosphorylate.

INTRODUCTION

The CaM-kinase family has recently been expanded through the cloning of two new members: CaM-kinases I (125) and IV (129, 348, 395). Members of this family have strong sequence homology in their NH_2-terminal kinase catalytic domains, significant homology in their central regulatory regions that encompass their autoinhibitory and CaM-binding domains, and variable COOH-terminal regions that can specify subunit association and/or subcellular localization. CaM-kinase II, the most extensively characterized member of the family, is an oligomeric enzyme which is particularly abundant in neural tissues (reviewed in (8)). It can phosphorylate a large number of proteins, and its activity is regulated by autophosphorylation. The substrate specificities of the other members of the CaM-kinase family are not entirely clear, but they appear to be more restricted than for CaM-kinase II. The role of regulatory autophosphorylation of the other members of the CaM-kinase family are also not well established.

CaM-kinase IV may be an important regulatory protein kinase since its activity has been reported to be strongly enhanced by autophosphorylation (352, 396). Several laboratories have purifed CaM-kinase IV from brain and thymus (349, 350), and its cDNA and predicted amino acid sequences have been determined (128, 129, 348, 380). The purified kinase is a monomer that exhibits relatively slow (30-60 min) autophosphorylation on a serine residue. The mechanism of autophosphorylation (intra- vs. intermolecular) (352, 396) and site(s) of autophosphorylation (351, 352) are controversial. However, several laboratories (352, 396) have reported that this Ca^{2+}/CaM-dependent autophosphorylation gives up to a 10-fold increase in total activity (assayed in the presence of Ca^{2+}/CaM) as well as an increase in Ca^{2+}-independent activity (assayed in the presence of EGTA). An increase in total CaM-kinase IV activity may be physiological since activation of the CD3 receptor in Jurkat cells produces a 17-fold increase in total activity of CaM-kinase IV (355). Thus, regulation of CaM-kinase IV may be significantly different

than that of CaM-kinase II which undergoes extremely rapid (10-30 sec) intramolecular Ca^{2+}/CaM-dependent autophosphorylation on T^{286} resulting in increased Ca^{2+}-independent but not total kinase activity (8).

Another intriguing characteristic of CaM-kinase IV is its significant nuclear localization (356). Nuclear localization is of potential importance since several reports strongly link CaM-kinases to Ca^{2+}-dependent transcriptional regulation (157, 169, 174). It is clear that CaM-kinase II can be involved in transcriptional regulation through phosphorylation of C/EBPß (174), but its role in transcriptional regulation through the CRE/CREB system is unclear even though CaM-kinase II can phosphorylate CREB on Ser^{133} (157, 164). It is known that phosphorylation of Ser^{133} is required for transcriptional activation by CREB (163). We (Chapter II, 403) and others (158, 165) have shown that the CaM-kinase inhibitor KN-62 (379) can strongly suppress Ca^{2+}-dependent transcription of a CRE-reporter gene or of several endogenous immediate early genes. Thus, it is important to determine which member(s) of the CaM-kinase family may be involved in transcriptional regulation.

To more fully understand the regulation of CaM-kinase IV and its role in transcriptional activation through phosphorylation of CREB, the biochemical properties of the purified CaM-kinase IV expressed in the baculovirus have been compared to those of CaM-kinase II expressed in the same system. We have focused on the role of regulatory autophosphorylation, inhibition by KN-62, and the ability of CaM-kinase IV to mediate transcriptional activation in COS-1 cells and to phosphorylate CREB *in vitro*.

EXPERIMENTAL PROCEDURES

Baculovirus Expression of CaM-Kinases

CaM-kinase IV cDNA from mouse brain, kindly provided by Dr. James Shikela (Univ. of Colorado Medical School) (380), was inserted into Not I/XbaI cut PVL1392 baculovirus transfer vector. CaM-kinases IV and II were expressed in the baculovirus expression system and purified on CaM-Sepharose as previously described (397) with the following modifications for CaM-kinase IV: prior to CaM-Sepharose, the 100,000g supernatant of CaM-kinase IV was batch-treated with phosphocellulose (CaM-kinase IV does not bind) to remove proteases; a 2M NaCl wash on CaM-Sepharose was used; and 1 mM benzamidine was added to the elution and storage buffers.

CaM-kinase Activity Assays

CaM-kinases IV and II were assayed at 30°C for 10 and 1 min, respectively, in 50mM HEPES, pH 7.5, 10mM magnesium acetate, 400 µM [γ-^{32}P] ATP (500-1200 cpm/pmol), 60 µM syntide-2 (or as indicated in the legend) and either 0.5mM Ca^{2+}/1 µM CaM (total kinase activity) or 1mM EGTA (Ca^{2+}-independent kinase activity) in a final volume of 25µl. All assays were initiated by addition of kinase diluted appropriately in 50mM HEPES, pH 7.5, 2mg/ml BSA and 10% ethylene glycol. ^{32}P-incorporation was determined by spotting 15 µl aliquots onto Whatman P81 as described (397). The inhibition experiments with KN-62 were performed as described above except that 150 nM of CaM and 50 nM of each kinase was used, and the reaction was initiated by adding a mixture of Mg^{2+}, [γ^{32}-P]ATP and CaM. All assays were performed in triplicate.

Autophosphorylation of CaM-kinases

Autophosphorylation was performed at 25°C in 50mM HEPES, pH 7.5 containing 10 mM magnesium acetate, 1mM Ca^{2+}/1.5 µM CaM, 1 mg/ml BSA, 400 µM ATP, and 1 µM

CaM-kinase II or 2 μM CaM-kinase IV. The reactions were terminated after 20 min by a 1:5 dilution into stopping buffer (50mM HEPES, pH 7.5, 10% ethylene glycol, 1mg/ml BSA, and 3mM EDTA). Each kinase was then diluted appropriately prior to activity measurements.

CREB phosphorylation and peptide mapping

Two previously described methods were used for bacterial expession of CREB and for its purification (156, 398). Essentially identical results were obtained with both preparations. CREB was phosphorylated *in vitro* by 50 nM of baculovirus-expressed CaM-kinase IV or 20 nM of CaM-kinase II in the conditions described above for the assays. As a control, CREB was also phosphorylated with 20 nM catalytic subunit of cAMP-kinase (PKA) using 50 mM of KH_2PO_4, pH 6.8, 10 mM magnesium acetate, 400 μM [γ-^{32}P]ATP, and 1 mM EGTA. The reactions were initiated by the addition of the kinases and after 1 min for CaM-kinase II and PKA or 10 min for CaM-kinase IV, 15 μl were removed and spotted on phosphocellulose papers (399). For the two-dimensional peptide mapping, recombinant CREB protein (1 μg) was phosphorylated *in vitro* as described above except that 50 nM of baculovirus-expressed CaM-kinase II or PKA were used. The reactions were initiated with kinase and incubated for 45 minutes at 30°C unless indicated otherwise.Two-dimensional peptide mapping was performed as described (232). 1000 cpm (determined by Cherenkov measurements) of the tryptic ^{32}P-CREB peptides were loaded on a cellulose plate for electrophoresis (25 min at 1 kv) using the Hunter Thin Layer Electrophoresis system (HTLE-7000, CBS Scientific Co.). Chromatography was then performed for 10 hrs using n-butanol:pyridine: acetic acid:water in volume ratios of 0.375: 0.25: 0.075: 0.30. The ^{32}P-peptides were detected by autoradiography. For $^{32}PO_4$-incorporation, CREB (10 μM) was phosphorylated as described above, and at the designated times, aliquots were removed and spotted on phosphocellulose papers. For the HPLC peptide mapping, CREB was selectively ^{32}P-labeled on site 1 using PKA as

described above for the two dimensional peptide mapping, or on site 2 using first PKA and cold ATP (50 μM) for 20 minutes followed by CaM-kinase II and [γ-^{32}P]ATP for 60 minutes. CREB was then precipitated three times with 10 % trichloracetic acid, washed extensively with ether, and air-dried. ^{32}P-CREB was dissolved in 100 mM Tris (pH 7.5) and digested overnight with 1 mg/ml trypsin. Samples were applied to a Beckman C18 (Ultrasphere ODS) column equilibrated in 0.1% trifluoroacetic acid (TFA). the column was washed for 10 minutes in 0.1% TFA before applying a linear gradient of 0 to 50% acetonitrile over 60 minutes. Fractions of 1 ml were collected and counted for radioactivity by Cerenkov radiation.

Phosphoamino Acid Analysis

Aliquots of the ^{32}P-peptides used for two-dimensional peptide mapping were lyophilized, resuspended in 200 μl of 6 N HCl, and hydrolyzed at 110°C for 60 min. After resuspension in pH 1.9 buffer (0.585 M formic acid, 1.36 M glacial acetic acid), phosphoamino acid standards were added and two dimensional mapping was performed as described (232).

Phosphatase treatment of CREB

CREB was selectively ^{32}P-labeled on site 1 or 2 as described above. The ^{32}P-CREB was heated at 65°C for 10 minutes to inactivate the kinases. It has previously been shown that this heat treatment does not effect the ability of CREB to bind to the CRE (156). The ^{32}P-CREB was diluted 2-fold in phosphatase buffer (100 μM $MnCl_2$ and 0.01% β-mercaptoethanol for phosphatase 1 and 2B; 50 mM Tris-HCl for phosphatase 2A), and the reaction was initiated by addition of the indicated concentration of phosphatase. Aliquots of the reaction containing 0.08 μg of CREB were spotted on phosphocellulose papers, and the papers were washed, dried and remaining radioactivity was determined as described above for the kinase assay. Phosphatases 1, 2A and 2B were kindly provided by

Drs. Ernest Lee (Univ. Miami), David Brautigan (Brown Univ.) and Brian Perrino (Vollum Institute), respectively.

Cell culture and transfections

COS-1 cells were maintained in Dulbecco's Modified Eagle Medium containing 10% FBS. Cells were subcultured in 60 mm dishes 12 hrs before transfection. The cells were then transferred to serum-free medium and treated with a mixture of DNA and 20 μg of LipofectAce reagent (GIBCO-BRL) in 1.5 ml medium. The cells were transfected with 5 μg of an expression vector for either the DNA-binding domain of the yeast transcriptional activator GAL4 or a CREB-GAL4 fusion protein (398) and 5 μg of a reproter plasmid containing 5 copies of a GAL4 DNA binding site upstream of a minimal promoter and the luciferase coding sequence (5XGAL4-TATA-Luciferase) and 5 μg of plasmids encoding constitutively-active forms of either PKA, CaM-kinase II or CaM-kinase IV. The PKA expression vector has been described previously (400). Expression vectors for constitutively-active CaM-kinase II and CaM-kinase IV were prepared by insertion of truncated cDNAs encoding residues 1-290 of CaM-kinase II and 1-313 of CaM-kinase IV in place of the globin cDNA in the RSV-globin expession vector (401). After incubation of the DNA mixture for 5 hrs., 3 ml of medium containing 20% FBS was added and the incubation was continued for another 20-26 hrs. The cells were collected, lysed and luciferase activity measured as described (402).

Other materials

The [γ-^{32}P]ATP was fron New England Nuclear Dupont. All other chemicals were from standard commercial sources.

RESULTS

Expression and characterization of CaM-kinase IV

Since CaM-kinases II and IV are members of the same protein kinase family, it is of interest to compare their physical, catalytic and regulatory properties. For this purpose, the α isoform of CaM-kinase IV was expressed using recombinant baculovirus in Sf9 cells and purified using CaM-Sepharose affinity chromatography analogous to our expression and purification of CaM-kinase II α subunit (397). The purified α CaM-kinase IV migrated on SDS/PAGE as a 63kDa species (not shown), and it eluted as a single peak on FPLC gel permeation with an apparent M_r of approximately 60-67kDa (Table 1). These results indicate that the expressed CaM-kinase IV α, like the purified brain CaM-kinase IV α (349), is monomeric compared to the oligomeric structure of CaM-kinase II (8). Of several peptides tested as substrates for CaM-kinase IV, syntide-2 and autocamtide were the best (Table 1). Peptide γ, reported to be a specific substrate for purified brain CaM-kinase IV (349), was barely phosphorylated by expressed CaM-kinase IV. The specific activity of expressed CaM-kinase IV was 0.1-0.2 μmol/min/mg using 40 μM syntide-2 as substrate. This value is the same as that reported for the purified brain CaM-kinase IV (349) but is approximately 100-fold less than for expressed CaM-kinase II (10 to 20 μmol/min/mg). The K_m of expressed CaM-kinase IV for syntide-2 (336 μM), however, was substantially higher than that reported (349) for purified brain CaM-kinase IV (2-5 μM). Of several proteins tested as substrates for CaM-kinase IV, synapsin I was the best with a phosphorylation rate about 10% that of CaM-kinase II.

There is strong sequence homology within the CaM-binding domains of CaM-kinases II and IV, and half-maximal activation of CaM-kinase IV was achieved with the same concentration of CaM (150 nM CaM at a Ca^{2+} concentration of 0.1 mM) as was required for activation of CaM-kinase II under similar assay conditions (Table 1). The CaM-kinase

inhibitor KN-62 interacts with CaM-kinase II competitively with Ca^{2+}/CaM (379), and since the CaM-binding domains of CaM-kinases IV and II are highly conserved, one might predict that KN-62 would inhibit CaM-kinase IV similar to CaM-kinase II. Figure 1 shows that KN-62 had very similar IC_{50}s (0.5-2 μM) for CaM-kinases IV and II. Because of the nuclear localization of CaM-kinase IV (356) and the involvement of CaM-kinases in Ca^{2+}-dependent transcriptional activation, we also tested CREB as a substrate for CaM-kinases IV and II (Table 1). More characteristics of CREB phosphorylation will be given later.

Autophosphorylation and activation of CaM-kinase IV

One of the most unique characteristics of CaM-kinase II is its rapid autophosphorylation on T^{286} which converts the enzyme to a Ca^{2+}-independent kinase (8). Several laboratories (351, 352) have reported that purified brain CaM-kinase IV catalyzes slow autophosphorylation of a Ser attaining a stoichiometry of approximately 1 mol ^{32}P per mol subunit. This autophosphorylation is reported to be associated with up to a 10-fold increase in total CaM-kinase IV activity and a substantial increase in Ca^{2+}-independent activity (352, 396). When expressed CaM-kinase IV was incubated under the autophosphorylation conditions described here, only 0.02-0.1 mol ^{32}P per mol subunit was incorportated at 30 min and no changes in total or Ca^{2+}-independent kinase activities were detected (Fig. 2A). CaM-kinase II autophosphorylation was performed in parallel as a positive control, and the characteristic increase in Ca^{2+}-independent activity was observed (Fig. 2B). Prolonged autophosphorylation for 2-3 hours does give up to a 2-fold increase in total activity (434) but this is unlikely to be physiologically important. Furthermore, the kinase was also preincubated with protein phosphatase 1 or 2A in an attempt to remove any endogenous phosphate prior to autophosphorylation in the presence of microcystin-LR, but these variables had little effect on the stoichiometry of phosphorylation or activity of the kinase. Thus, in spite of numerous attempts to detect physiologically significant

autophosphorylation-dependent changes in the activity of expressed CaM-kinase IV, we have been unable to do so.

Transcriptional activation by CaM-kinase IV

KN-62 inhibits Ca^{2+}-dependent transcription of genes containing a CRE in their promoters (158, 165) and it also inhibits both CaM-kinases II and IV, so it was important to determine whether CaM-kinase IV can stimulate transcription through the CRE-binding protein, CREB. This was tested by co-transfecting COS-1 cells with a GAL4-CREB fusion construct, a GAL4-luciferase reporter gene, and expression vectors for constitutively-active kinases. The transfected CaM-kinases II and IV were truncated at residues 290 and 313, respectively, whereas the catalytic subunit of PKA was used. These CaM-kinase truncations are in analogous positions in the two kinases, just NH_2-terminal of the CaM-binding domain. Truncation of CaM-kinase II at residue 290 has been demonstrated to result in a fully active kinase which can stimulate transcription through phosphorylation of C/EBPß (169, 174). Fig. 3 shows that transfection with CaM-kinase IV resulted in a 3-fold elevation in luciferase expression whereas transfection with CaM-kinase II gave little stimulation over the control plasmid. Transfection with PKA stimulated luciferase expression 6 to 7-fold. This experiment was repeated three times with similar results each time.

Phosphorylation of CREB

Since transfected CaM-kinase IV gave transcriptional activation through CREB, we further characterized the *in vitro* kinetics and specificities of CREB phosphorylation by the three protein kinases. CaM-kinase IV phosphorylated CREB (Table 1 and Fig. 4A), and only Ser residues were ^{32}P-labeled by CaM-kinase IV (Fig. 4B). Fig. 5 shows the concentration-dependency for CREB phosphorylation by CaM-kinase IV, CaM-kinase II and PKA. From this data kinetic values could be calculated, and all three protein kinases

had similar K_m values for CREB phosphorylation, but the V_{max} values for CaM-kinase II and PKA were about 40-fold greater than the V_{max} of CaM-kinase IV (Table 2). This may explain why CaM-kinase IV was less potent at transcriptional activation compared to PKA (Fig. 3), but the transcriptional impotency of CaM-kinase II is paradoxical from this data.

We next determined the site-specificities of the three protein kinases for CREB phosphorylation. Two-dimensional peptide maps of CREB phosphorylated by PKA, CaM-kinase IV and CaM-kinase II are illustrated in Fig. 6. When CREB-341 was phosphorylated by CaM-kinase IV, the same peptide map (Fig. 6D) was obtained as with PKA phosphorylation of CREB (Fig. 6A). This site was identified as Ser^{133} (site 1) by using a mutant of CREB in which Ser^{133} was mutated to Ala (S133A) (Figs. 6B and 6E). CaM-kinase II, however, catalyzed incorporation of 2 mol $^{32}PO_4$ per mol CREB (Fig. 7, insert) equally into two major sites (Fig. 6G; Fig. 7, dotted line). One site was S^{133} since it was absent 1) in the S133A CREB mutant (Fig. 6H) and 2) after prior phosphorylation with PKA and non-radioactive ATP (Fig. 7, dashed line). Phosphoamino acid analysis showed that site 2 was also a serine as CREB 341 and its mutant S133A showed only phosphoserine (Fig. 4C and 4D). Since Ser^{98} in CREB is a predicted consensus CaM-kinase II phosphorylation site, we examined phosphorylation of CREB-327 which is lacking 14 residues, including Ser^{98}, due to alternative splicing. Both sites of CaM-kinase II phosphorylation were present in CREB-327 (Fig. 6I), thus eliminating Ser^{98} as the second CaM-kinase II site. In other experiments, we have varied the CaM-kinase II concentration or time of phosphorylation to obtain a stoichiometry of phosphorylation around 1 mol ^{32}P per mol CREB, and both sites were phosphorylated equally. Similarly, higher concentration of CaM-kinase IV were used, and in every case S^{133} was the major single phosphorylation site.

Dephosphorylation of CREB

The observation that CaM-kinase II was ineffective at transcriptional activation through the CREB system (Fig. 3) in spite of catalyzing phosphorylation of Ser^{133} in CREB, suggests that the second CaM-kinase II phosphorylation site 2 may be a negative determinant. If so, dephosphorylation of site 2 could play an important regulatory role. Therefore, we surveyed the major protein phosphatases for their abilities to differentially dephosphorylate sites 1 and 2 in CREB. To label site 1 (Ser^{133}), CREB was phosphorylated by PKA using [γ-^{32}P]ATP. Site 2 was specifically ^{32}P-labeled by phosphorylating site 1 with PKA and cold ATP followed by addition of CaM-kinase II and [γ-^{32}P]ATP. HPLC peptide mapping confirmed that under these conditions only site 2 was ^{32}P-labeled (Fig. 7). Fig. 8 illustrates the rates of *in vitro* dephosphorylation of sites 1 or 2 by protein phosphatases 1, 2A or 2B (calcineurin). In these experiments we determined the lowest concentration of each phosphatase that gave rapid dephosphorylation of site 1. Our results show that phosphatases 1 and 2A (1-10 μM) gave similar rates of dephosphorylation of sites 1 and 2. Calcineurin (phosphatase 2B, 25μM) was also very active towards site 1 but significantly less so towards site 2. However, 100μM of calcineurin did dephosphorylate site 2 (not shown).

DISCUSSION

Over the past five years increasing evidence has accumulated that members of the CaM-kinase family are involved in Ca^{2+}-dependent transcriptional regulation. The best characterized systems are the Ca^{2+}-dependent expression of the immediate early gene (IEG) *c-fos* (158), the glucagon gene (165), and sequences containing the CaM-kinase II responsive element (CaMRE): 5'-AAATGTAGTCTTATGCAATACACTTGTAGTCTTG TAGTCTTGCAACA-3' (169). The *c-fos* and glucagon genes utilize the CREB/CRE enhancer system (157, 165) whereas the CaMRE binds C/EBPß (174). Ca^{2+}-dependent phosphorylation of C/EBPß (174) and transcription of the glucagon gene (165) are strongly suppressed by the cell permeable CaM-kinase inhibitor KN-62. We have recently demonstrated that KN-62 strongly inhibits the Ca^{2+}-dependent, but not the forskolin/cAMP- or phorbol ester-dependent, transcription of the three IEGs *c-fos*, NGFI-A and NGFI-B (Chapter II and 403). However, it is not clear which member(s) of the CaM-kinase family mediate these various Ca^{2+}-dependent transcriptional activations. CaM-kinase II, a multifunctional member of the CaM-kinase family, is known to phosphorylate CREB on Ser^{133} (157, 164) and to give a small enhancement of *in vitro* transcription using a CRE-chloramphenicol acetyltransferase (CAT) reporter gene (164) but no stimulation of a CRE-reporter gene in pituitary cells (169). CaM-kinase II also phosphorylates Ser^{276} in C/EBPß and gives a 60-fold stimulation of expression of a CaMRE-reporter gene (174). Thus, evidence for a role of CaM-kinase II in transcriptional regulation through the C/EBPß system is strong, but CaM-kinase II appears to be quite weak in stimulating transcription *via* the CRE/CREB system. The recent discovery of CaM-kinase IV (348) and its significant nuclear localization (356) prompted us to assess its potential role in CRE/CREB-mediated transcription.

The Sf9 cell expressed CaM-kinase IV was similar to the purified brain CaM-kinase IV in physical properties, but it differed significantly in certain catalytic properties (Table 1).

The most important difference is that expressed CaM-kinase IV, unlike the purified brain kinase (352, 396), was not significantly regulated by autophosphorylation. This lack of autophosphorylation-dependent changes in CaM-kinase IV activity has also been reported for bacterially-expressed CaM-kinase IV (353) and for Sf9-expressed CaM-kinase IV. While this manuscript was in preparation, a paper appeared which reported characterization of baculovirus-expressed CaM-kinase IV (354). Their characterization of CaM-kinase IV properties are very similar to ours including the lack of regulatory autophosphorylation. The most likely explanation for the observed "autophosphorylation" of purified brain CaM-kinase IV is a contamination by another protein kinase which can phosphorylate and activate CaM-kinase IV. Indeed, a brain extract is able to catalyze a large activation of expressed CaM-kinase IV (353, 434). This *in vitro* activation of CaM-kinase IV by another protein kinase is consistent with the observation that activation of the CD3 receptor in Jurkat cells produces a 17-fold increase in CaM-kinase IV activity (355). Presumably the CD3 receptor stimulation activates a protein kinase (CaM-kinase IV kinase) which mediates phosphorylation and activation of CaM-kinase IV. If the V_{max} of CaM-kinase IV were increased 15- to 20-fold by "CaM-kinase IV kinase", then CREB would be a very good substrate (Table 2).

The existence of an apparent cascade activation system for CaM-kinase IV is of potential importance with regards to involvement of CaM-kinase IV in transcriptional regulation, the major focus of this investigation. Our results demonstrate that CaM-kinase IV, like PKA, phosphorylated CREB predominantly (i.e, greater than 90%) on Ser^{133} (Fig. 4A, 4B and Fig 6D, 6E, 6F). Furthermore, co-transfection of CaM-kinase IV with a GAL4-CREB construct and a GAL4-reporter gene resulted in a 3-fold enhancement of transcription (Fig. 3). This transcriptional response to CaM-kinase IV was obtained with a truncated form of CaM-kinase IV which is constitutively active in the absence of Ca^{2+}/CaM (354). It is not known if this truncated CaM-kinase IV can be further activated by phosphorylation with CaM-kinase IV kinase. If so, it is possible that an even stronger

transcriptional response would be obtained if this cascade system were operative. The weaker transcriptional response elicited by CaM-kinase IV compared to PKA may be due to the low V_{max} for CaM-kinase IV phosphorylation of CREB. However, the ability of CaM-kinase IV to enhance transcription through the CRE/CREB system appears to be somewhat variable depending on the cell type. In GH3, PC12 and JEG-3 cells CaM-kinase IV can give up to a 4- to 5-fold transcriptional stimulation through the GAL4-CREB system (1). In these cells CaM-kinase II is also ineffective at stimulating transcription through the CREB/CRE system, but CaM-kinase II does elicits a 4-fold induction of the prolactin gene, confirming that the transfected CaM-kinase II is active. Our observed lack of transcriptional activation by co-transfection with CaM-kinase II is consistent with previous reports that CaM-kinase II gives no enhancement of transcription through the CRE/CREB system (169), but this is surprising since CaM-kinase II is a good catalyst for phosphorylation of CREB (Fig. 5 and Table 2). In fact, the kinetic parameters for phosphorylation of CREB by CaM-kinase II are comparable to those of PKA and much better than those of CaM-kinase IV. However, we now report that CaM-kinase II catalyzed equivalent phosphorylation of Ser^{133} and a second site in CREB (Fig. 6H). This second site is not Ser^{98}, a consensus CaM-kinase II site, since both sites were present in CREB327 which lacks Ser^{98} (Fig. 6J). Previous studies on the phosphorylation of CREB by CaM-kinase II have identified S^{133} as the only or the major site of phosphorylation (157, 164). Equivalent phosphorylation of two sites in CREB has been consistently observed in our laboratory for three years using either baculovirus expressed α CaM-kinase II, β CaM-kinase II or purified brain CaM-kinase II (not shown) and identification of the phosphorylation sites by two methods, two-dimensional peptide mapping (Fig. 6) or mapping by reverse phase HPLC (Fig. 7). This second CaM-kinase II phosphorylation site in CREB has recently been identified as S^{142}, and its function as a negative determinant in CREB-mediated transcriptional activation has been established (1). The existence of phosphorylation sites which are negative regulators of transcription have been identified in

other transcription factors such as c-Jun (57), c-Fos (55, 56), and more recently another CRE-binding protein, CREMt, has been shown to probably exhibit negative regulation by phosphorylation (404).

Our results do not rule out the possibility that in COS cells, the ability of CaM-kinase IV to activate transcription through the CRE/CREB system is mediated indirectly through the Ca^{2+}-sensitive adenylate cyclase (122). In that case PKA would be an essential component of Ca^{2+}-regulated transcription as suggested by Ginty *et al.* (211).

Since this second CaM-kinase II phosphorylation site in CREB acts as a negative determinant for transactivation, selective dephosphorylation of site 2 relative to site 1 by a protein phosphatase could enhance the ability of CaM-kinase II to activate transcription through CREB phosphorylation. Therefore we determined the relative rates of dephosphorylation of sites 1 and 2 in CREB by the most prominent protein phosphatases, phosphatases 1, 2A and 2B (calcineurin). Previous studies using selective phosphatase inhibitors such as okadaic acid have indicated that phosphatases 1 (263) or 2A (267) may regulate dephosphorylation of CREB. Our *in vitro* results (Fig. 8) showed that phosphatases 1 and 2A at 1-10 μM dephosphorylate sites 1 and 2 at similar rates. Phosphatase 2B, or calcineurin, exhibited similar potency (25 μM) towards site 1 but not for site 2. It is known that calcineurin can exist in the nucleus, but its physiological significance for dephosphorylation of CREB has not been explored. Elevations of intracellular Ca^{2+} promote phosphorylation of CREB (156), presumably by activation of CaM-kinase II or IV. If calcineurin and CaM-kinase II were activated simultaneously, CaM-kinase II would phosphorylate sites 1 and 2 whereas calcineurin could selectively dephosphorylate site 1, thereby ensuring an inactive CREB. If CaM-kinase IV phosphorylated site 1 in CREB under conditions of elevated intracellular Ca^{2+}, activated calcineurin would tend to oppose this phosphorylation. We have recently shown in PC12 cells that inhibition of calcineurin by FK506 has no effect on Ca^{2+}-dependent transcription of *c-fos*, but FK-506 enhances transcription of NGFI-A and strongly suppresses the

transcription of NGFI-B (Chapter II and 403). This complex pattern of differential effects of calcineurin inhibition on three immediate early genes, all of which contain CRE or CRE-like elements in their promoters, may not be consistent with a simple mechanism of action of calcineurin to dephosphorylate CREB. We are now in the process of determining if the calcineurin effects on the promoters of these three immediate early genes are mediated through their CRE/CREB systems or other enhancer elements.

Acknowledgements: We would like to thank Jane Richards for providing some of the CREB and Dr I. P. Shapiro for his help and advice with the 2-D peptide mapping system. We also would like to thank Drs. Richard Maurer and Richard Goodman for valuable discussions and suggestions during the course of these studies and for critical reading of the manuscript.

TABLE 1. Properties of CaM-kinases IV and II. The Comparisons of the kinase activities toward peptide and protein substrates are given as pmol/min and, in parenthesis, as a percentage compared to 500 μM syntide-2 for each kinase. All reactions were initiated by addition of 20 nM CaM-kinase II or 50 nM CaM-kinase IV.

	Expressed CaM-kinase II	Expressed CaM-kinase IV
Gel permeation elution (kD)	600	67
EC_{50} CaM (nM)	100	150
Km Syntide-2 (μM)	21	336
Vmax Syntide-2 (μmol/min/mg)	19.8	0.7
	Kinase activity	Kinase activity
Peptides	pmol/min	pmol/min
Syntide-2: 50 μM	123.3 (106%)	3.8 (21.5%)
500 μM	116.1 (100%)	17.7 (100%)
Autocamtide: 50 μM	79.6 (69%)	4.1 (23%)
500 μM	77 (66%)	19.6 (111%)
Peptide γ: 50 μM	0.63 (<1%)	0.1 (<1%)
500 μM	5.3 (4.6%)	0.7 (4%)
PKC 19-36: 50 μM	12 (10%)	0.6 (3.5%)
500 μM	15 (12.9%)	1.7 (9.5%)
Proteins		
Synapsin I: 50 μg/ml	12.5 (10.8%)	0.4 (2.2%)
300 μg/ml	32.3 (27.8%)	3.5 (19.8%)
MBP: 50 μg/ml	2.4 (2.1%)	0.1 (<1%)
300 μg/ml	0.68 (<1%)	0.3 (1.7%)
CREB: 50 μg/ml	9.25 (8%)	0.312 (1.8%)
300 μg/ml	14.4 (12.4%)	0.683 (3.9%)

Table 2. Kinetic constants for CREB phosphorylation using the data from Figure 5.

CREB	CaM-kinase II	CaM-kinase IV	PKA
Km (μM)	1	2.5	4.7
Vmax (μmol/min/mg)	1.14	0.025	0.82

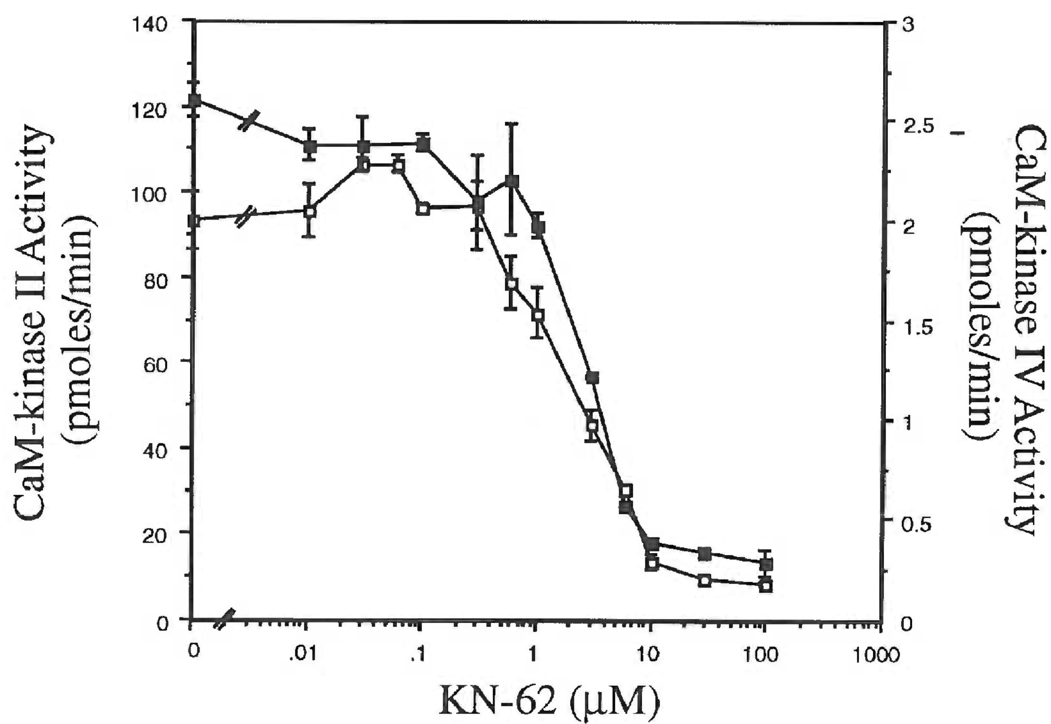

Figure 1. Inhibition of CaM-kinases IV and II by KN-62. 50 nM of purified CaM-kinase II (filled squares) and IV (open squares) were assayed for total kinase activities (see Experimental Procedures) in the presence of 150 nM CaM and the indicated concentrations of KN-62.

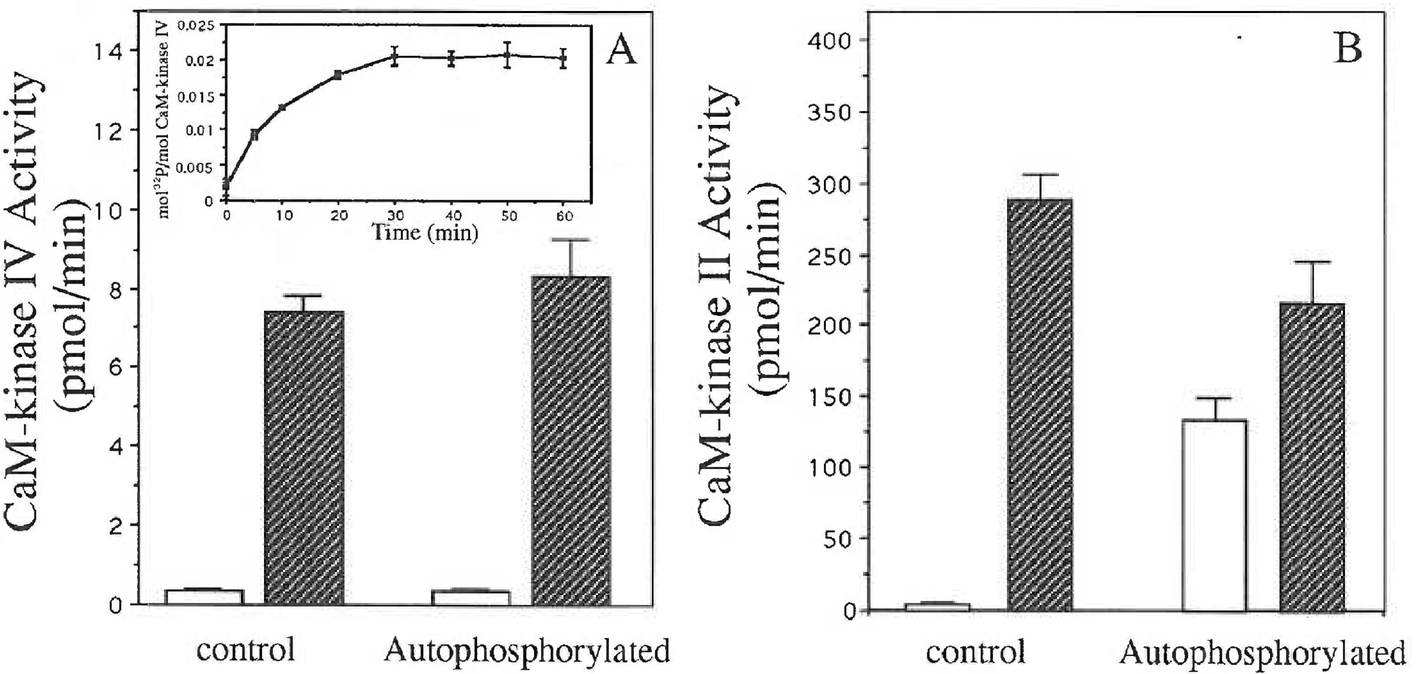

Figure 2. Effects of autophosphorylation on CaM-kinases IV and II. CaM-kinases IV (2 μM, panel A) and II (1 μM, panel B) were subjected to standard Ca^{2+}/CaM-dependent autophosphorylation conditions (see Experimental Procedures) for 60 min (kinase IV) or 30 min (kinase II). Aliquots of CaM-kinase IV were spotted on phosphocellulose papers at the times indicated in the insert for quantitation of ^{32}P-incorportation. After 30 min of autophosphorylation, the reaction was terminated with a 5-fold dilution into stopping buffer, and Ca^{2+}-independent (open bars) or total (cross-hatched bars) kinase activities were then assayed at a final concentration of 50 nM for kinase II and 100 nM for kinase IV. Controls lacked ATP during the autophosphorylation.

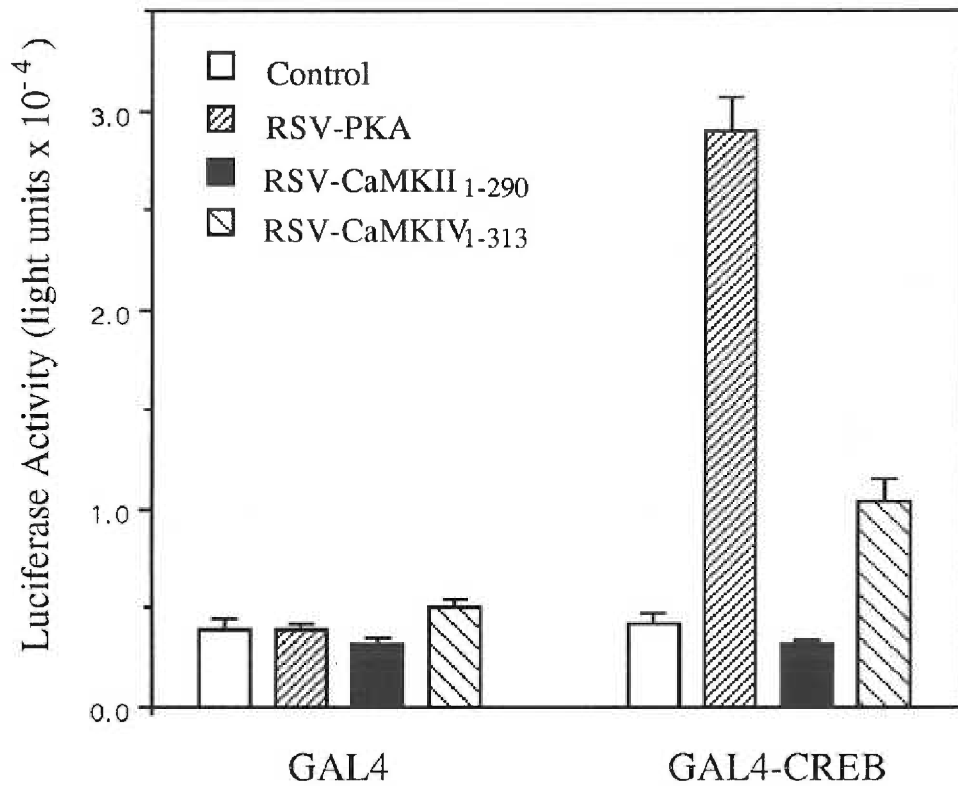

Figure 3. Transcriptional efficiencies of CaM-kinases IV and II. COS-1 cells were transfected with 5 mg of an expression vector for either GAL4 (1-147) or a GAL4-CREB fusion protein and 5 mg of a reporter gene containing 5 copies of a GAL4 DNA-binding site upstream of a minimal promoter and the luciferase coding sequence (5XGAL4-TATA-luciferase) and 5 mg of either BSSK(-) as control or 5 mg of expression vectors for constitutively-active forms of either PKA, CaM-kinase II or CaM-kinase IV.

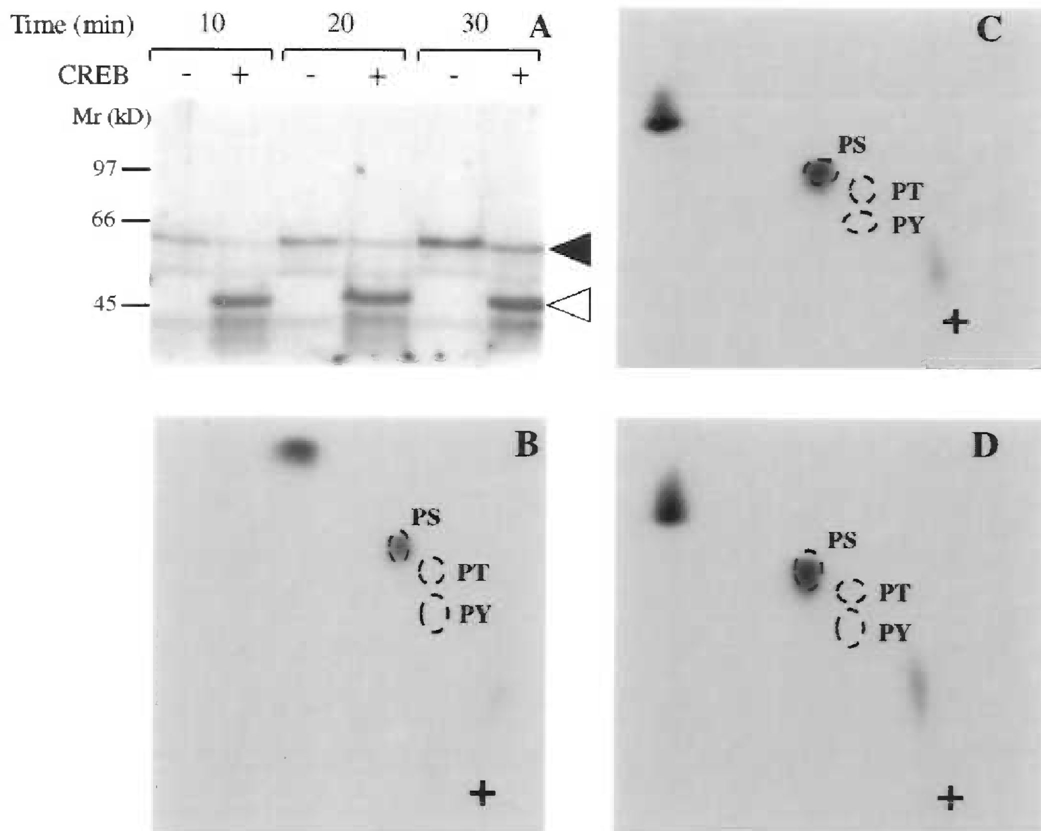

Figure 4. Phosphoamino acid analyses of ^{32}P-CREB phosphorylated by CaM-kinases IV and II. Panel A. Purified CREB341 (1 μg) was phosphorylated by CaM-kinase IV (50 nM) for the indicated times. Aliquots were removed and subjected to SDS/PAGE and autoradiography. Controls (CREB -) contained CaM-kinase IV but no CREB. Open and filled arrows mark the positions of ^{32}P-CREB and autophosphorylated CaM-kinase IV, respectively. Panels B-D. The ^{32}P-CREB341 (panels B and C) or mutant S133A (panel D), phosphorylated by CaM-kinase IV (panel B) or CaM-kinase II (panels C and D), was excised from the SDS/PAGE and subjected to tryptic digestion and partial acid hydrolysis (6 N HCl at 110°C for 1 hr) prior to two-dimensional separation of the ^{32}P-amino acids. The positions of standard phosphoamino acids (phosphoserine, PS; phosphothreonine, PT; phosphotyrosine, PY) are indicated.

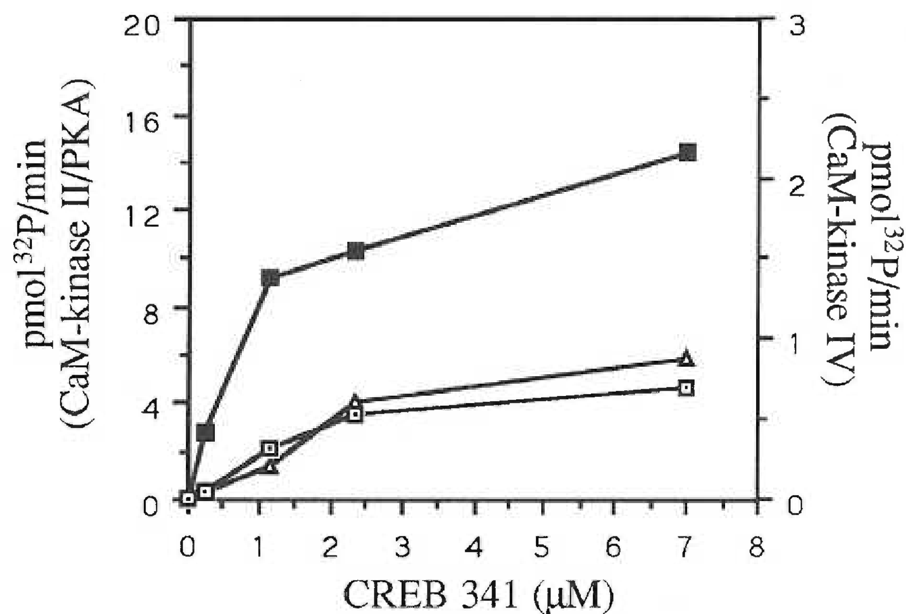

Figure 5. Concentration dependency of CREB phosphorylation. The indicated concentrations of purified CREB341 were phosphorylated by 20 nM CaM-kinase II (filled squares) or PKA (open triangles) or by 50 nM CaM-kinase IV (open squares) (see Experimental Procedures). Aliquots of the reaction were spotted on phosphocellulose papers at 1 min (CaM-kinase II and PKA) or 10 min (CaM-kinase IV) and processed to quantitate the initial rates of CREB phosphorylation. Note the different scale used for CaM-kinase IV phosphorylation.

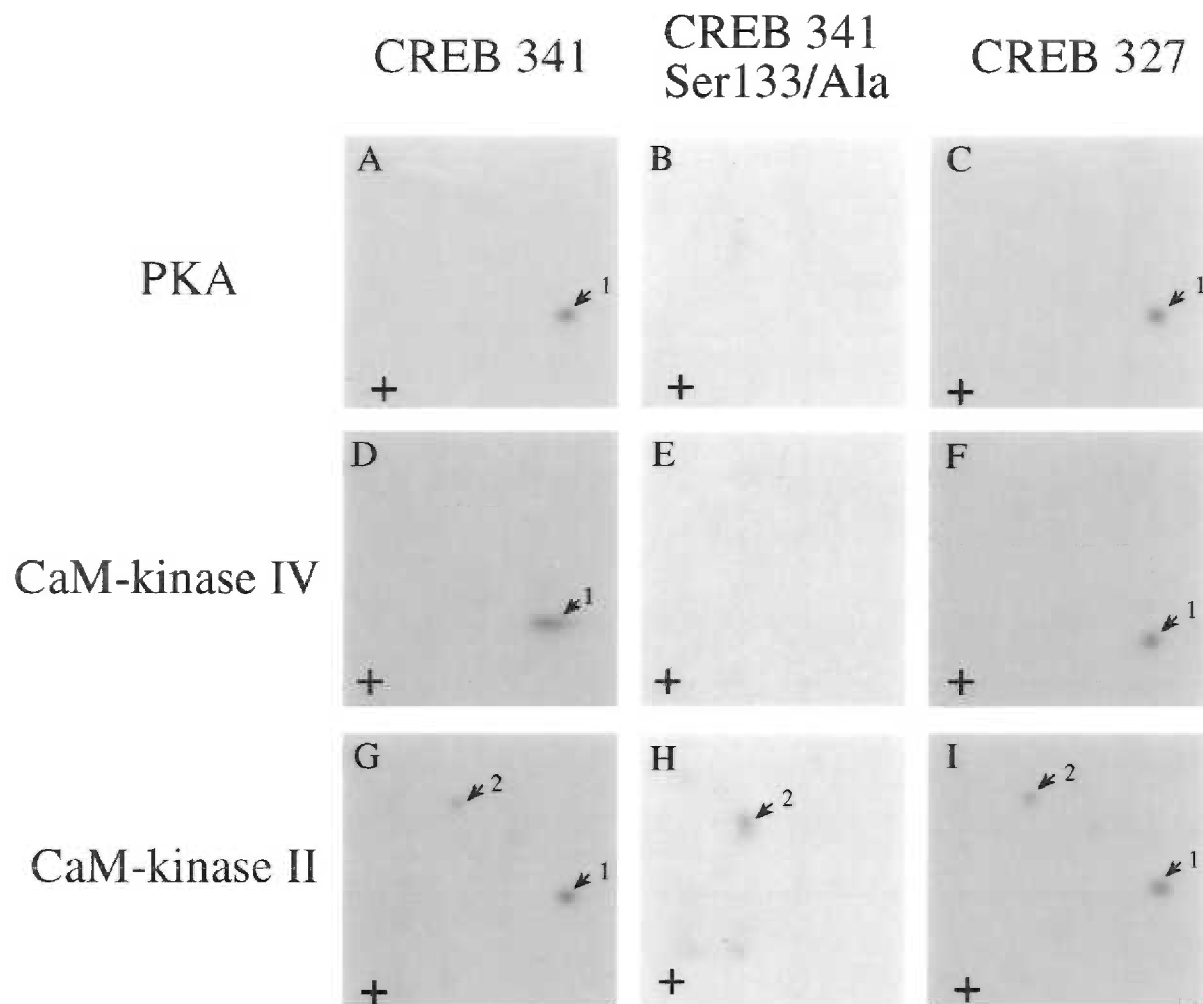

Figure 6. Two-dimensional peptide mapping of phosphorylation sites in CREB. 1 to 2 µg of CREB (CREB341, CREB341 mutant S133A, or CREB327) were ^{32}P-labeled by 50nM of PKA, CaM-kinase IV or CaM-kinase II as indicated. The ^{32}P-CREB was excised from SDS/PAGE, subjected to tryptic digestion and two-dimensional peptide mapping (see Experimental Procedures). The two major CREB phosphorylation sites 1 and 2 are indicated by the arrows, and the origin is denoted by the cross.

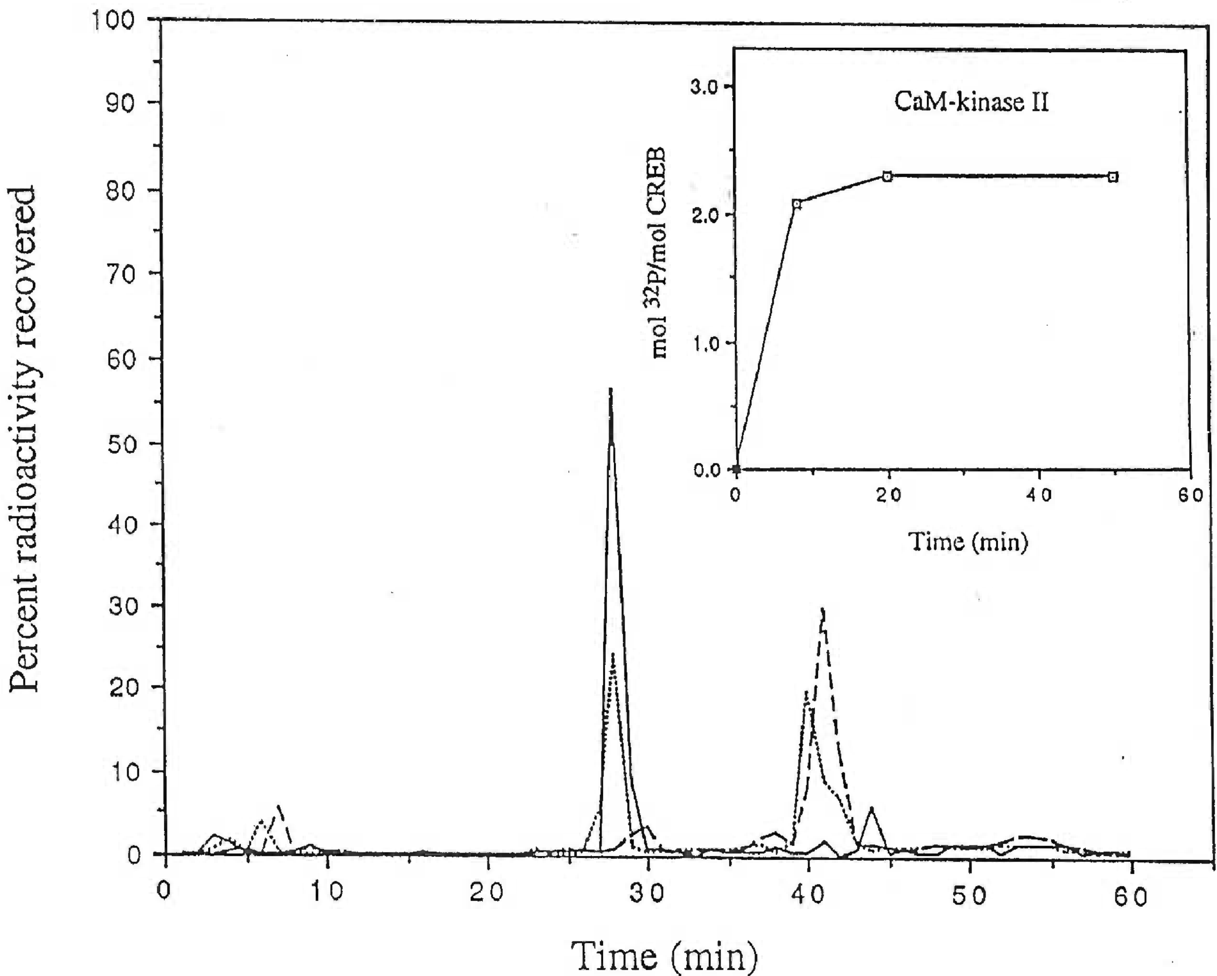

Figure 7. HPLC peptide mapping of CREB phosphorylation sites. CREB was phosporylated with either 50 nM CaM-kinase II (see inset) or PKA. The 32P-CREB was processed and digested with trypsin as described in Experimental Procedures. The 32P-peptides derived from CREB phosphorylated by CaM-kinase II (dotted line) or PKA (solid line) were subjected to reverse phase HPLC mapping. Tryptic peptides from a third sample of CREB that was phosphorylated by 50 nM PKA and non-radioactive ATP for 20 min followed by 50 nM CaM-kinase II and [gamma-32P]ATP for 60 min. are shown by the dashed line. The small peak prior to 10 min is residual [gamma-32P]ATP.

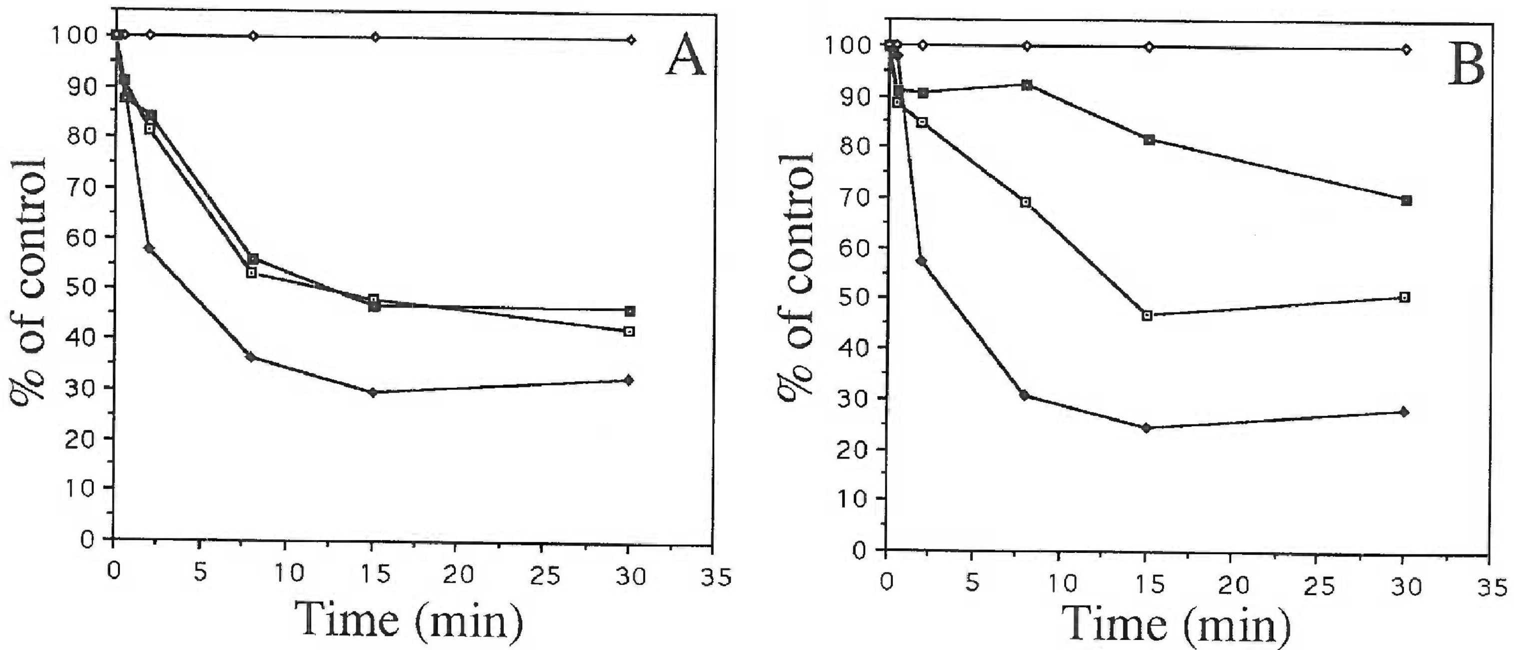

Figure 8. Dephosphorylation of CREB sites 1 and 2 by protein phosphatases. CREB341 was phosphorylated by PKA and 75 mM [gamma-^{32}P-]ATP (site 1, panel A) or 50 µM cold ATP followed by addition of CaM-kinase II and 100 mM [gamma-^{32}P]ATP (site 2, panel B). The ^{32}P-CREB was heated (65°C for 10 min) to inactivate the kinases, briefly centrifuged to removed denatured protein, and aliquots of the supernatant were incubated with protein phosphatase 1 (10nM, filled diamonds), 2A (2 nM, open squares), or 2B (25nM, filled squares) (see Experimental Procedures). At the indicated times aliquots of the reaction were spotted on phosphocellulose papers to determine remaining ^{32}P-CREB. CaM-kinase II autophosphorylation did not represent more that 5% of the total radioactivity (not shown). The control reaction lacking any phosphatase was defined as 100% at each time point (open diamonds).

IV

DISCUSSION

The requirement for calcium during muscle contraction has been known for about 100 years, but the role of calcium as a key signalling molecule in nearly every eukaryotic cell has only been discovered in the last 20 years. Calcium regulates many different biological phenomena that persist from milliseconds to hours or days after the change in calcium concentration. Recently the role of calcium in the regulation of long term events in the nervous system has received attention from neuroscientists. The transcriptional regulation of immediate early genes (IEGs) that encode transcription factors which are responsible for the control of late gene activity is a major system by which the cell can efficiently direct its long-term response to external stimuli. The pioneering work of Michael Greenberg's laboratory at Harvard Medical School, dissecting the pathways used by calcium to regulate IEG's transcription, demonstrated the importance of calcium-regulated transcription in the nervous system. Membrane depolarization induces IEGs transcription in PC12 cells (16), and the cAMP-responsive element (CRE) has been shown to mediate *c-fos* induction by membrane depolarization (155, 156) through phosphorylation of the CRE binding protein (CREB) (157). The kinase responsible for CREB phosphorylation in response to membrane depolarization was not identified. Three different laboratories (157, 164, 165) suggested that CaM-kinase II, which has an overlap in substrate specificity with PKA, could be responsible for CREB phosphorylation in cells. Indeed, CaM-kinase II is an excellent catalyst for CREB phosphorylation *in vitro*. However, Michael Rosenfeld's group demonstrated that an expression vector encoding a constitutively-active form of CaM-kinase II did not activate the transcription of a reporter gene under the control of a CRE, suggesting that CaM-kinase II was not the correct candidate (169). The work of this

thesis has been focused on trying to understand the role of CaM-kinases and phosphatase in the regulation of IEGs by calcium and calmodulin.

The calcium regulation of IEGs is mediated by CaM-kinase(s)

We have demonstrated in PC12 cells that transcriptional induction by calcium (membrane depolarization or calcium ionophore treatment) of the three IEGs *c-fos*, NGFI-A and NGFI-B was blocked by KN-62 (Chapter II, Figs. 2 and 4B), a cell permeable synthetic compound that had been previously characterized as a specific inhibitor of CaM-kinase II (379). KN-62 inhibited CaM-kinase II activation in PC12 cells (Chapter II, Fig. 1 and 379) but had no effect on the stimulation of the three IEGs by forskolin, which activates adenylate cyclase and results in the activation of PKA, or by TPA, which directly activates PKC. This result demonstrated the specificity of KN-62 for the calcium pathway (Chapter II, Fig. 4A). The effect of KN-62 was due to direct inhibition of CaM-kinase and not due to any nonspecific effect on the calcium flux during the calcium ionophore treatment (392). We also clearly demonstrated that treatment of PC12 cells with the calcium ionophore A_{23187} did not activate PKA (Chapter II, Fig.3). This result rules out any effect due to activation of a Ca^{2+}/CaM-dependent adenylate cyclase (122) or activation of PKA due to proteolytic activation by the calcium-activated protease, calpain (213). Our results using KN-62 suggest that in addition to *c-fos*, other IEGs such as NGFI-A and NGFI-B are positively regulated by CaM-kinase(s).

Role of CaM-kinase II and CaM-kinase IV in the activation of transcription through CREB

In order to establish which CaM-kinase(s) were responsible for the calcium induction of the IEGs, we compared the ability of two multifunctional CaM-kinases to regulate transcription. CaM-kinase IV, a recently cloned and monomeric CaM-kinase (128-130), has been localized in the nucleus and is therefore an attractive candidate for transcriptional regulation. We expressed CaM-kinase IV in the baculovirus system and, as

well as others (354), characterized the properties of this enzyme *in vitro* (Chapter III, Table I). One of the major differences between CaM-kinase II and CaM-kinase IV is that CaM-kinase II generates a Ca^{2+}-independent form upon rapid autophosphorylation, whereas the autophosphorylation of CaM-kinase IV was very weak and generated little Ca^{2+}-independent activity (Chapter III, Fig.2). We showed that *in vitro* KN-62 inhibited CaM-kinase IV as well as CaM-kinase II with an IC_{50} between 0.5 and 2 μM (Chapter III, Fig. 1), demonstrating that KN-62 could equally inhibit CaM-kinase II and CaM-kinase IV in PC12 cells. Thus the induction of *c-fos*, NGFI-A and NGFI-B by calcium might be mediated by at least 2 different CaM-kinases: CaM-kinase II and CaM-kinase IV.

Two laboratories reported that CaM-kinase II phosphorylates CREB at Ser133 (157, 164), the site that triggers transcriptional activation when phosphorylated by PKA (247), so we tested wether CaM-kinase IV could phosphorylate CREB *in vitro*. Indeed, CaM-kinase IV phosphorylated CREB (Chapter III, Figs. 4A and 4B), and this phosphorylation only occured at Ser133 as determined by two-dimensional peptide mapping (Chapter III, Figs. 6D, 6F). Furthermore, when Ser133 in CREB was mutated to Ala, CREB was not phosphorylated by CaM-kinase IV (Fig. 6E). Kinetic experiments determined that the Kms of PKA, CaM-kinases II and IV for CREB were very similar (between 1 and 5 μM), and that the Vmax of CaM-kinase II and PKA were also very similar (about 1 μmol/min/mg) whereas the Vmax of CaM-kinase IV for CREB was about 40 fold lower (0.025 μmol/min/mg) (Chapter III, Fig.5 and Table II). We have now shown that if CaM-kinase IV was activated by the CaM-kinase IV kinase, purified in our laboratory (434), the Vmax for CREB is increased 25 to 35 fold compared to the one obtained with nonactivated CaM-kinase IV (assayed in the presence of Ca^{2+}/CaM), whereas the Km was similar to the one observed for CaM-kinase II and PKA (not shown). Thus, the Vmax of the "activated" CaM-kinase IV (activated by CaM-kinase IV kinase) for CREB is similar to the one observed with CaM-kinase II and PKA. Phosphorylation of CREB by "activated" CaM-kinase IV (not shown) occurs, like phosphorylation by "non-

activated" CaM-kinase IV, exclusively on Ser133 (Chapter III, Table II). Surprisingly, we found using 2D or HPLC peptide mapping that the α isoform of CaM-kinase II catalyzed the phosphorylation of a second Ser residue distinct from Ser133 (Chapter III, Figs. 4C-D, 6G-I and 7). Similar results were obtained with the β isoform as well as with the brain purified enzyme (not shown). These results prompted us, in collaboration with Peiqing Sun in Dr. Maurer's laboratory, to test the ability of CaM-kinase II and IV, to activate transcription through CREB phosphorylation. Using vectors containing cDNAs encoding constitutively-active, truncated forms of CaM-kinase II and IV (residues 1 to 290 for CaM-kinase II and 1-313 for CaM-kinase IV) as well as the catalytic subunit of PKA with the CREB-GAL4 system in COS-1 cells, we observed an 8-to 10-fold stimulation of transcription through CREB phosphorylation by PKA and a 3-fold stimulation by non-activated CaM-kinase IV (Chapter III, Fig.3). CaM-kinase II gave no induction of transcription in this system (Chapter III, Fig.3) suggesting that its second phosphorylation site in CREB is a dominant negative determinant for activation of transcription. Dr. Maurer's laboratory has now determined that this second site phosphorylated by CaM-kinase II is Ser142 and that it is a dominant negative determinant (1). The sequence surrounding Ser142 : -I-L-N-D-L-S*-S-D-A-P-G- is different from the established consensus phosphorylation sequence for CaM-kinase II, confirming that factors others than Arg in position -3 can be important in determining phosphorylation by CaM-kinase II. It is interesting to point out that the protein vimentin is phosphorylated by CaM-kinase II on Ser82 and that the recognition motif for this phosphorylation is also -S*-X-D- (335). These results demonstrate that CaM-kinase II and CaM-kinase IV, like PKA, can phosphorylate CREB at Ser133 but that only CaM-kinase IV and PKA can activate transcription through CREB phosphorylation (see figure 1 this chapter, p.92). CaM-kinase IV is to date the best candidate for the activation of transcription by calcium through CREB phosphorylation (as CaM-kinase I does not appear to be nuclear).

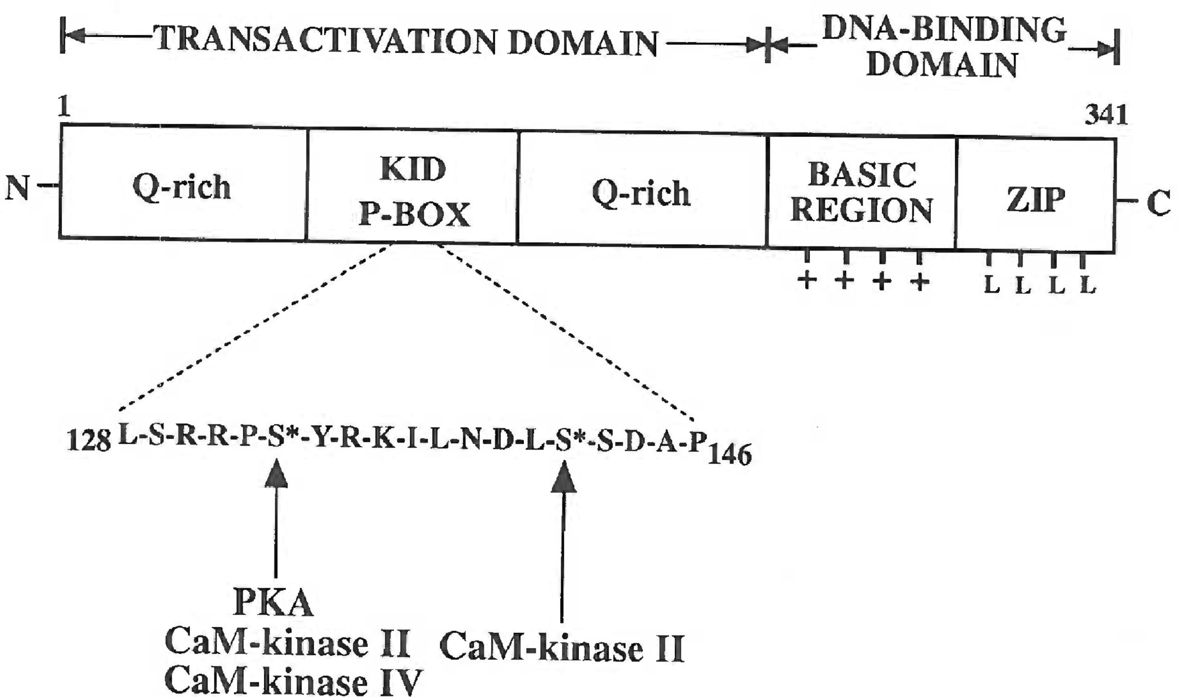

Figure 1. Positive and negative regulation of transcription by CaM-kinases through the phosphorylation of CREB. CaM-kinase II and CaM-kinase IV, like PKA, can phosphorylate CREB at Ser133 but only CaM-kinase IV and PKA can activate transcription through CREB phosphorylation. The inability of CaM-kinase II to activate transcription is due to the phosphorylation of Ser142.

A role for CaM-kinase II in regulating transcription through phosphorylation of other transcription factor has been reported. For example, CaM-kinase II can stimulate transcription of the prolactin gene (169) and inhibit transcription of IL-2 (405). Moreover, a new isomer of CaM-kinase II, δ_B, has recently been cloned from rat heart and demonstrated to be differentially targeted to the nucleus (406) in contrast to other isoforms which are excluded from the nucleus (381, 406). An 11-amino acid insert present in the variable domain of δ_B-CaM-kinase II appears to be responsible for its nuclear localization (406). Another mechanism that could permit CaM-kinase II nuclear translocation is its proteolytic digestion, which renders it monomeric and constitutively active (as the form used in our experiments). However this mechanism has only been demonstrated *in vitro* (399). Thus, the presence of CaM-kinase II and CaM-kinase IV in the nucleus of the same cell is possible. However, if CaM-kinase II was present in the nucleus and phosphorylated CREB, transcription through the CRE would not be activated because of Ser142 phosphorylation. Numerous genes containing a CRE in their promoters are induced by calcium. This suggests that if Ser142 is phosphorylated in cells, a mechanism must exist to dephosphorylate this residue. However, a selective dephosphorylation *in vivo* of Ser142 over Ser133 by a protein phosphatase could result in the indirect activation of transcription by CaM-kinase II. To test this hypothesis, we monitored *in vitro* the ability of the three major protein phosphatases PP1, PP2A and PP2B or CaN to dephosphorylate Ser133 and Ser142. None of the three protein phosphatases tested preferentially dephosphorylated Ser142 over Ser133 (Chapter III, Fig.8). We still cannot rule out that *in vivo* an as yet unknown protein phosphatase can efficiently dephosphorylate Ser142 over Ser133. Another possible mechanism would be that Ser142 is constitutively phosphorylated in resting cells by the basal level of CaM-kinase II and that calcium would simultaneously trigger Ser133 phosphorylation and Ser142 dephosphorylation resulting in CREB activation.

The mechanism by which phosphorylation of Ser142 prevents transcriptional activation, even when Ser133 is phosphorylated, is not known. One possibility is that the interaction between CREB phosphorylated on Ser133 and its co-activator CBP (CREB-binding protein) (271) is prevented or modified by the phosphorylation of Ser142. A change in the conformation of CREB due to Ser142 phosphorylation could also be responsible for a modified interaction of CREB with CBP or with the basal transcriptional machinery. Negative transcriptional regulation by CaM-kinase II is not unprecedented as Nghiem *et al.* recently demonstrated that CaM-kinase II blocks transcriptional initiation of the IL-2 gene. This block could be mediated by phosphorylation of the transcription factor NF-AT (405). In addition, these investigators demonstrated that a constitutively-active form of CaM-kinase IV did not activate a reporter gene under the control of the IL-2 gene promoter. These results in addition to the ones obtained for the regulation of transcription through CREB phosphorylation demonstrate that CaM-kinase II and CaM-kinase IV can regulate transcription positively and/or negatively and that regulation of transcription by these enzymes is not limited to the CRE/CREB and CEBP/β system.

Our results provide a model in which calcium-induced-CREB phosphorylation can positively or negatively regulate CRE-driven transcription depending on the CaM-kinase involved. The tissue distribution of CaM-kinase II and IV, their subcellular localization, cellular concentration, affinity for CREB phosphorylation as well as the dephosphorylation of Ser142 are critical factors in this model that determine whether calcium activates or prevents transcriptional activation through CREB phosphorylation.

Role of Calcineurin in the regulation of IEG's transcription by calcium

Our results using FK506 and CsA suggest that CaN regulates the transcription of NGFI-A and NGFI-B in PC12 cells (Chapter II, Figs. 5A-B and 6). PC12 is a cell line used as a model to study certain neuronal-specific phenomena such as differentiation and neurite outgrowth. Our observations constitute the first report of the involvement of CaN in

transcriptional regulation in a neuronal model. In immune cell lines CaN positively regulates the transcription of IL-2 and IL-4 through the transcription factor NF-AT(p) (190, 191, 407, 408). In the kidney CsA decreases transcription of the PEPCK, gene; this effect is organ-specific as hepatic PEPCK gene expression is unaffected (409). CaN also positively controls the adenovirus major late promoter in lymphoma cells (410). Our results extend this list of regulated genes to NGFI-A and NGFI-B. In PC12 cells CaN regulates NGFI-A negatively and NGFI-B positively, suggesting that in the case of NGFI-A CaM-kinase(s) and CaN effects would antagonize each other whereas they would synergize calcium-induction of NGFI-B. This is consistent with the recent report by Schwaninger *et al.* using pancreatic islet cells. They have demonstrated that membrane depolarization relies not only on CaM-kinase(s) (165) but also on CaN for activation of CREB/CRE-mediated gene transcription using transfection of reporter fusion genes (196). In this study FK506 and CsA inhibited the activation (by membrane depolarization) of a reporter gene under the control of 350 bp of the glucagon gene promoter in the pancreatic islet cell line HIT-T15. The same results were obtained with a reporter gene under the control of the glucagon, the choriogonadotropin and the somatostatin CREs. In this sytem overexpression of CaN renders the activation by membrane depolarization resistant to the effects of CsA and FK506. These studies suggest that in pancreatic islet cells CaM-kinase(s) and CaN could mediate positive regulation by calcium through CREB and the CRE. Such an activation mechanism is possible for NGFI-B because of the presence of the two AP-1 like sequences within -22 to -86 that can function as CREs. However, in the case of CaN our results with *c-fos* in PC12 cells (Chapter III, Figs. 5A-B and 6) as well as the reports of others in T cells (179, 411, 412) demonstrate that CaN is probably not involved in the calcium regulation of the endogenous *c-fos* gene. These different results could be explained by the fact that the *c-fos* promoter can be activated by calcium through responsive elements other than the CRE (156, 158, 198, 381, 413, 414).

Simultaneous activation of CaM-kinase(s) and CaN by calcium results in the activation of NGFI-A, suggesting that the balance between the kinase and the phosphatase is in favor of CaM-kinase(s). One possible explanation for the predominant effect of CaM-kinase(s) is that both CaM-kinase(s) and CaN will be activated differentially in response to an increase of intracellular calcium (415). CaN has a 100-to 1000-fold greater affinity for CaM than CaM-kinase II or IV; thus, the phosphatase will be preferentially activated during the ascending phase of the Ca^{2+} transient when the calcium is relatively low, and in particular if calmodulin is not in large excess. The CaN activation will be rapid and will only be dependent on the rate of association between CaN and the Ca^{2+}/CaM complex. CaN is immediately inactivated when the calcium concentration goes down. When activated, CaN will trigger a protein phosphatase cascade through PP1. CaN dephosphorylates I-1 which then no longer inhibits PP1 and thereby promotes the dephosphorylation of multiple proteins. As the calcium concentration further increases, the concentration of Ca^{2+}/CaM complex also increases until it is sufficient to activate CaM-kinases. Upon activation, CaM-kinase II undergoes a rapid autophosphorylation on Thr286 that renders it partially Ca^{2+}-independent (8). Similarly, CaM-kinase IV becomes independent of calcium and CaM after activation by CaM-kinase IV kinase (434). When the calcium concentration decreases, CaN is immediately inhibited whereas CaM-kinase II and IV activities are sustained. Protein phosphatases will eventually inactivate the two kinases. Thus a short and transient CaN activation compared to a slower but more prolonged CaM-kinase activation could possibly explain why the kinase effect is predominant. Our results suggest that the low calcium induction of NGFI-A, compare to NGFI-B and *c-fos*, could be due in part to the inhibitory effect of CaN.

The activation by the same second messenger (i.e., Ca^{2+}/CaM) of a kinase and a phosphatase that will have opposite effects is intriguing but not unique. A model of synaptic plasticity suggesting that CaM-kinase II and CaN have opposite effects on the efficiency of synaptic transmission has been developed (416). Furthermore, very recently

CaM-kinase II and calcineurin have been shown to antagonize each other in the calcium regulation of the IL-2 gene. In T cells and Jurkat cells, calcineurin positively regulates IL-2 transcription while CaM-kinase II blocks the stimulation of Il-2 by co-treatment with calcium ionophore and phorbol esters or by the constitutively-active form of CaN and PMA. CaN and CaM-kinase II effects seem to be mediated by the same promoter regions of IL-2, notably the NF-AT binding site (405). There are 12 CaM-kinase II consensus phosphorylation sites in NF-AT, and it will be important to determine if CaM-kinase II effects are mediated through the phosphorylation of NF-AT. To date CaM-kinase(s) and CaN have not been found to have any substrates in common. Direct interaction between CaM-kinases and CaN may be possible, although in the study on IL-2 expression constitutively-active constructs were used ruling out phosphorylation of CaN in its calmodulin binding domain. Although Ca^{2+}-independent CaM-kinase II can phosphorylate the COOH-terminus of the CaM-binding domain in CaN, this has little or no known regulatory effect (417). Calcineurin, however, does not dephosphorylate Thr286 in CaM-kinase II.

In general, previously reported effects of CaN on transcription have been positive effects (80, 190, 191, 407, 408, 410). We demonstrate that NGFI-B is also positively regulated by CaN. However, we provide evidences here, that in PC12 cells CaN can regulate transcription negatively. The substrate(s) of CaN responsible for the negative regulation of NGFI-A remain to be characterized.

Role of PKA in the regulation of IEG's transcription by calcium

Numerous sites of interaction between the Ca^{2+} and cAMP signalling system have been characterized such as Ca^{2+}/CaM-dependent adenylate cyclase, CaM-dependent phosphodiesterase and voltage-sensitive Ca^{2+} channels that can be regulated through direct phosphorylation by PKA. One other interaction site between the two pathways is CREB phosphorylation and activation as described and demonstrated above. We have shown that

an increase of intracellular calcium concentration does not have any effect on the state of activation of PKA in PC12 cells, but a basal level of PKA activity is required for membrane depolarization-activated *c-fos* and NGFI-A (211) and calcium ionophore treatment to stimulate *c-fos*, NGFI-A and NGFI-B transcription (our results not shown). One possibility is that the basal level of PKA is required to phosphorylate the protein phosphatase 1 inhibitor, inhibibitor-1 (I-1). Phosphorylated I-1 interacts with PP1 and inhibits the activity of this protein phosphatase. PP1 has a wide substrate specificity and can dephosphorylate many proteins important for calcium induction of transcription such as CREB (263). Thus, the role of the basal activity of PKA could be to keep I-1 phosphorylated in order to inhibit PP1 and prevent the dephosphorylation of CREB or any other protein(s) that could be important for transcriptional activation by calcium. However our results suggest that it is not the case for NGFI-B (Chapter II, Fig.7).

Sheng *et al.* demonstrated that in addition to converging on CREB for *c-fos* activation, calcium and cAMP have synergistic effects on transcription through the CaRE (156). This result is intriguing, particularly if activation of each of these two second messenger pathways converges on Ser133-phosphorylation in CREB. This synergistic effect suggests that in addition to Ser133 phosphorylation, Ca^{2+} and cAMP may also modulate mutually exclusive domains, either on CREB or on another CaRE/CRE-binding protein. In T cells a mechanism by which Ca^{2+} and cAMP induce *c-fos* synergistically has been proposed by Lee *et al.* (413). They demonstrated that calcium regulates *c-fos* expression through 2 discrete molecular mechanisms. The first one, as already described above, targets an element immediately adjacent to the SRE and triggers increased initiation of *c-fos* transcription. The second mechanism regulates the elongation of transcripts initiated in response to other signals, and this mechanism may account for the observed synergy between calcium and cAMP in T cells. It will be interesting to examine in PC12 cells the regulation of *c-fos* elongation in response to calcium stimulation. The presence of such a mechanism in PC12 cells, would suggest that cAMP as well as calcium could enhance

transcriptional initiation (through different promoter elements), but only calcium would increase elongation resulting in a synergistic effect on the total transcription when both second messengers are present.

Promoter sequences requirement for the induction of c-fos, NGFI-A and NGFI-B by calcium and other second messengers

The absence of a typical CRE sequence in the promoters of NGFI-A and NGFI-B suggests that their induction by calcium could be mediated by pathways other than the one using CREB. Once again, the numerous studies on the calcium regulation of *c-fos* are very informative. The CRE/CREB is not the only pathway used by calcium to regulate *c-fos*: in HeLa cells, for example, CRE-containing *c-fos* promoters do not respond to calcium (418) and several laboratories have shown that the serum response element (SRE) can mediate calcium-activated transcription of *c-fos* (158, 198, 413). In addition, Matthews *et al.* showed that expression vectors encoding truncated, constitutively-active forms of CaM-kinase II and CaM-kinase IV can activate a reporter gene under the control of the *c-fos* promoter that contains the CRE and the SRE motifs; the authors state that the SRE mediates the induction by CaM-kinase II (381). This result is particularly interesting regarding a potential role for the transcription factor C/EBPβ in the Ca^{2+} induction of *c-fos*. Phosphorylation of C/EBPβ within its leucine zipper (at Ser276) by CaM-kinase II results in transcriptional activation in GH3 cells (174). Metz *et al.* have shown that upon an increase of intracellular cAMP in PC12 cells, C/EBPβ is phosphorylated and translocated from the cytosol to the nucleus (419). Once in the nucleus, C/EBPβ can activate *c-fos* transcription by interacting with specific regions of the SRE (419). It is intesting to note that in T cells the same site in the SRE mediates calcium induction of *c-fos* (413). To date the site of C/EBPβ phosphorylated in PC12 cells has not been determined; furthermore, PKA does not seem to directly phosphorylate C/EBPβ (174). Thus, it will be important to

test if in PC12, Ca^{2+} activation of *c-fos* can be mediated by CaM-kinase II and/or CaM-kinase IV through C/EBPβ and the SRE.

In PC12 cells membrane depolarization and calcium influx also stimulate MEK and MAP kinases *via* activation of Ras (414). MAP-kinase and the MAP kinase-regulated ribosomal protein S6 kinase II (pp90rsk) can phosphorylate transcription factors that interact with the SRE and control *c-fos* expression (420-424).

In macrophages (199), myeloid leukemia cells (201) and T cells (413) Ca^{2+} has been demonstrated to not only increase *c-fos* transcriptional initiation but also to induce the relief of a transcription elongation block beyond the end of the first intron. In our nuclear run-on experiments we have used full length probes of each IEG, so we cannot differentiate between an effect of calcium on the initiation or on the elongation of transcription. Our results using KN-62 suggest that CaM-kinase(s) are involved in the activation of IEGs transcription by calcium. This induction could occur at the level of elongation by direct phosphorylation of the RNA polymerase, an associated elongation factor or a DNA-bound regulatory protein. Thus, in addition to activating transcription at the level of initiation through the CRE-CaRE/CREB or the SRE, CaM-kinases could also regulate the elongation of *c-fos* mRNA in PC12 cells. However, it remains to be established if this type of regulation by calcium also exists in the nervous system or is mainly specific for the immune system.

It remains to be determined which binding site(s) present in the promoters of NGFI-A and NGFI-B are responsible for the calcium induction of these genes. Analyses of deletion promoter mutants of NGFI-A and NGFI-B will give us a better idea of which responsive element mediate the calcium induction. The use of FK506/CsA or KN-62 will indicate if the induction by CaN and CaM-kinase(s) are mediated by the same promoter region and ultimately the same or different transcription factor(s).

V

CONCLUSION

Animals acquire new information about their environment through learning, but to retain this information they need memory. It has been shown that at least two different forms of memory exist. Short-term memory, lasting seconds to hours and long-term memory lasting days to years. As shown in invertebrates, short-term memory does not require new protein synthesis. Long-term memory has been shown to require expression of the same genes for proteins involved in short-term memory. Therefore, the covalent modifications used in short term memory (i.e., phosphorylation of ion channels) are likely to be important for long-term memory, as well.

Calcium, one of the most important second messengers that regulate synaptic transmission in the nervous system, controls long-term phenotypic changes of neuronal cells in part by regulating the expression of IEGs. The mechanisms by which calcium affect the activity of these genes is not clearly understood. We have investigated the involvement of two protein kinases (CaM-kinase II and IV) and a protein phosphatase (CaN) that are activated by calmodulin in the calcium induction of the three IEGs *c-fos*, NGFI-A and NGFI-B. Our observations further the understanding of the role of each of these enzymes in the regulation of IEGs transcription by calcium and allowed us to propose a working model as described in the Discussion section that incorporates the results obtained by us and others.

It is interesting to note that in the case of NGFI-A induction by calcium, the model that we are proposing has striking similarities with an existing model for the regulation of synaptic strength. Long-term potentiation (LTP) in hyppocampus, which increases synaptic

strength for several hours, depends on phosphorylation of glutamate-gated ion channels by CaM-kinase II upon calcium influx into the postsynaptic dendritic spine (340, 425). Interestingly, long-term depression (LTD), the reversal of the process responsible for LTP, weakens synaptic strength and is blocked by inhibitors of calcineurin. Like LTP, LTD is input specific and requires activation of NMDA receptors as well as some rise in postsynaptic Ca^{2+}. Thus, NMDA receptor-mediated rise in Ca^{2+} is involved in both LTP and LTD. A model to explain these results has been proposed by Lisman (339) and updated recently by Malenka (114). In this model, a balance between the activity of CaM-kinase II and PP1 influences synaptic strength by controlling the phosphorylation state of some phosphoprotein(s), probably AMPA-type ion channels. As described in Chapter IV, PP1 activity is regulated by CaN through dephosphorylation of I-1. Small increases in calcium favor CaN activation which results in PP1 activation while larger rises are necessary for increasing CaM-kinase II activity. In our model NGFI-A is positively regulated by CaM-kinase(s) and negatively by CaN. The correlation between LTP/LTD regulation and the regulation of NGFI-A by calcium is further stengthened by a report where NGFI-A has been cloned from a highly sensitive differential complementary DNA cloning procedure to identify genes that may participate in long-term plasticity (84). NGFI-A has also been shown to be induced by synaptic activity that is responsible for long-term plasticity (426). The role that NGFI-A plays in defining long-term changes in neuronal cells is certainly to regulate the transcription of late genes that can be involved in the regulation of synaptic plasticity. A gene which expression is strongly upregulated in LTP is the one encoding CaM-kinase II. The mRNA levels for the α-isoform of CaM-kinase II, but not for the β-isoform, are doubled after induction of LTP (427). Furthermore, the mRNA for CaM-kinase II migrated to the distal dendrites (427), and it is known that translation of CaM-kinase II occurs in the dendrites (428, 429).

It is also very interesting to note that NGFI-B, which is strongly induced by calcium in neuronal cells, is required for apoptosis in T cells. As activation of glutamate

receptors is known to have a toxic effect in neurons, the role of NGFI-B in this toxicity remains to be examined.

The CREB/CRE system, as demonstrated by us and others, is very important in the regulation of IEGs by calcium. Two consecutive reports in the same issue of the journal *Cell* (430, 431) recently demonstrated that CREB is required for long-term but not for short-term memory in drosophila and mice, confirming similar results previously obtained in mollusks (432). This result suggests that CREB is probably responsible for the regulation of genes necessary for long-term memory (433). The role in long-term memory of other signalling pathways than cAMP, such as calcium, that use the CREB/CRE system to regulate transcription remains to be established. This is particularly important in regard to the fact that a specific increase of the mRNA for the α isoform of CaM-kinase II has been reported over the soma and the dendritic field of dentate granule cells following LTP induction (427).

However, a lot of questions remain to be answered. In the case of calcium regulation through CREB, it remains to be demonstrated whether *in vivo* phosphorylation of Ser142 occurs as well as the mechanism by which phosphorylated Ser142 prevents transcriptional activation. Also, the now very abundant literature on calcium regulation of transcription certainly suggests that different kinases or phosphatases could be responsible for the effects observed depending on which responsive element has a preponderant role for the calcium response in a given cell line. Obviously, tissue specificity and the context of the promoter elements are critical in determining the pathways used by calcium to regulate gene expression. The determination of the promoter sequence responsible for calcium induction of NGFI-A and NGFI-B will certainly be a great advance in understanding the pathways used by calcium to regulate the transcription of these genes. Finally, the role of transcriptional elongation compared to transcriptional initiation should be better characterized in neuronal cells as well as cells from the immune system.

REFERENCES

1. Sun, P., Enslen, H., Myung, P. S., and Maurer, R., A. (1994) *Genes & Dev.* **8**, 2527-2539.
2. Berridge, M. J. (1993) *Nature* **361**, 315-325.
3. Evered, D. (1986). Calcium and the Cell. CIBA foundation symposium, .
4. Nishizuka, Y. (1988) *Nature* **334**, 661-665.
5. Parker, P. J., Kour, G., Marais, R. M., Mitchell, F., Pears, C., Schaap, B., Stabel, S., and Webster, C. (1989) *Mol. Cell. Endocrinol.* **65**, 1-11.
6. Scott, J. D. (1991) *Pharmacol. Ther.* **50**, 123-145.
7. Taylor, S. S. (1989) *J. Biol. Chem.* **264**, 8443-8446.
8. Colbran, R. J., and Soderling, T. R. (1990) *Curr. Top. Cell. Regul.* **31**, 181-221.
9. Soderling, T. R. (1990) *J. Biol. Chem.* **265**, 1823-1826.
10. Scott, J. D., and Soderling, T. R. (1992) *Curr. Opin. Neurobiol.* **2**, 289-295.
11. Hanson, P. I., and Schulman, H. (1992) *Ann. Rev. Biochem.* **61**, 559-601.
12. Guerini, D., and Klee, C. B. (1991) *Adv. Prot. Phosphatase* **6**, 391-410.
13. Greenberg, M. E., Greene, L. A., and Ziff, E. B. (1985) *J. Biol. Chem.* **260**, 14101-14110.
14. Morgan, J. I., and Curran, T. (1986) *Nature* **322**, 552-555.
15. Lau, L. F., and Nathans, D. (1987) *Proc. Natl. Acad. Sci U. S. A.* **84**, 1182-.
16. Bartel, D. P., Sheng, M., Lau, L. F., and M.E., G. (1989) *Genes & Dev.* **3**, 304-313.
17. Barzilai, A., Kennedy, T. E., Sweatt, J. D., and Kandel, E. R. (1989) *Neuron* **2**, 1577-1586.
18. Merli, J. P., Isenberg, K. E., Russel, S. D., and Sanes, J. R. (1984) *J. Cell. Biol.* **99**, 332-335.

19. Castellucci, V. F., Kennedy, T. E., Kandel, E. R., and Goelet, P. (1988) *Neuron* **1**, 321-328.
20. Goldman, D., Breener, H. R., and Heinemann, S. (1988) *Neuron* **1**, 329-333.
21. Offord, J., and Catterall, W. A. (1989) *Neuron* **2**, 1447-1452.
22. Klarsfeld, A., Laufer, R., B., F., Devillers-Thiery, A., Dubreuil, C., and Changeux, J. P. (1989) *Neuron* **2**, 1229-1236.
23. Kelly, K., Cochran, B. H., Stiles, C. D., and Leder, P. (1983) *Cell* **35**, 603-610.
24. Greenberg, M. E., and Ziff, E. B. (1984) *Nature* **311**, 433-438.
25. Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K., and Tjian, R. (1987) *Science* **238**, 1386-1392.
26. Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988) *Cell* **54**, 541-552.
27. Kato, G. J., and Dang, C. V. (1992) *FASEB J.* **6**, 3065-3072.
28. Milbrandt, J. (1987) *Science* **238**, 797-799.
29. Sukhatme, V. P., Cao, X., Chang, L. C., Tsai-Morris, C. H., Stamenkovitch, D., Ferreira, P. C. P., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., Le Beau, M. M., and Adamson, E. D. (1988) *Cell* **53**, 37-43.
30. Lemaire, P., Relevant, O., Bravo, R., and Charnay, P. (1988) *Proc. Natl. Acad. Sci U. S. A.* **85**, 4691-4695.
31. Lim, R. W., Varnum, B. C., and Herschman, H. R. (1987) *Oncogene* **1**, 263-270.
32. Milbrandt, J. (1988) *Neuron* **1**, 183-188.
33. Hazel, T. G., Nathans, D., and Lau, L. F. (1988) *Proc. Natl. Acad. Sci U. S. A.* **85**, 8444-8448.
34. Ryseck, R.-P., Macdonald-Bravo, H., Mattei, M.-G., Ruppert, S., and Bravo, R. (1989) *EMBO J.* **8**, 3327-3335.
35. Varnum, B. C., Lim, R. W., and Herschman, H. R. (1989) *Oncogene* **4**, 1263-1265.

36. Nakai, A., Kharta, S., Sakurai, A., Toback, F. G., and DeGroot, L. J. (1990) *Mol. Endocrinol.* **4**, 1438-1443.

37. Curran, T., Abate, C., Cohen, D. R., Macgregor, P. F., Rauscher III, F. J., Sonnenberg, J. L., Connor, J. A., and Morgan, J. A. (1990) *Cold Spring Harbor Symposia on quantitative Biology* **LV**, 225-234.

38. Ransone, L. J., and Verma, I. M. (1990) *Annu. Rev. Cell. Biol.* **6**, 539-557.

39. Pfahl, M. (1993) *Endocrine Reviews* **14**, 651-658.

40. Radler-Pohl, A., Gebel, S., sachsenmaier, C., Konig, H., Kramer, M., Oehler, T., Streile, M., Ponta, H., Rapp, U., Rahmsdorf, h. J., Cato, A. C. B., Angel, P., and Herrlich, P. (1993) *Annals New York Academy of Sciences* **684**, 127-148.

41. Landschulz, W. H., Johnson, P. F., and Mcknight, S. L. (1988) *Science* **240**, 1759-1764.

42. Kouzarides, T., and Ziff, E. (1988) *Nature* **336**, 646-651.

43. Bos, T. J., Raucher, F. J. I., Curran, T., and Vogt, P. K. (1989) *Oncogene* **4**, 123-126.

44. Gentz, R., Rauscher, F. J. I., Abate, C., and Curran, T. (1989) *Science* **243**, 1695-1699.

45. O'Shea, E. K., Rutkowski, R., Stafford, W. F. I., and Kim, P. S. (1989) *Science* **245**, 646-648.

46. Schuermann, M., Neuberg, M., Hunter, J. B., Jenuwein, T., Ryseck, R. P., Bravo, R., and Muller, R. (1989) *Cell* **56**, 507-516.

47. Ransone, L. J., Visvader, J., Sassone-Corsi, P., and Verma, I. M. (1989) *Genes & Dev.* **3**, 770-781.

48. Turner, R., and Tjian, R. (1989) *Science* **244**, 1689-1694.

49. Nakabeppu, Y., Ryder, K., and Nathans, D. (1988) *Cell* **55**, 907.

50. Rauscher, R. J. I., Voulalas, P. J., Franza Jr, B. R., and Curran, T. (1988) *Genes & Dev.* **2**, 1687.

51. Cohen, D. R., and Curran, T. (1989) *Crit. Rev. Oncogen.* **1**, 65.

52. Zerial, M., Toschi, L., Ryseck, R. P., Schermann, M., Muller, M., and Bravo, R. (1989) *EMBO J.* **8**, 805.

53. Macgregor, P. F., Abate, C., and Curran, T. (1990) *Oncogene* **5**, 451-458.

54. Meijlink, F., Curran, T., Miller, A. D., and Verma, I. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4987-4991.

55. Ofir, R., Dwarki, V. J., Rashid, D., and Verma, I. M. (1990) *Nature* **348**, 80-82.

56. Tratner, I., Ofir, R., and Verma, I. M. (1992) **7**, 998-1006.

57. Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M., and Hunter, t. (1991) *Cell* **64**, 573-584.

58. Christy, B., and Nathans, D. (1989) **86**, 8737-8741.

59. Lemaire, P., Vesque, C., Schmitt, J., Stunnenberg, H., Frank, R., and Charnay, P. (1990) *Mol. Cell. Biol.* **10**, 3456-3467.

60. Cao, X., Koski, R. A., Gashler, A., Mckiernan, M., Morris, C. F., Hay, R. V., and Sukhatme, V. (1990) *Mol. Cell. Biol.* **10**, 1931-1939.

61. Ishii, S., Kadonago, J. T., Tjian, R., Brady, J. N., Merlino, G. T., and Pastan, I. (1986) *Science* **232**, 1410-1413.

62. Smith, R., Peters, G., and Kickson, C. (1988) *EMBO J.* **7**, 1013-1022.

63. Shtivelman, G., lifshitz, B., Gale, R. P., Roe, B. A., and Canaani, E. (1986) *Cell* **47**, 277-284.

64. Sorger, P. K., and Pelham, H. R. B. (1987) *EMBO J.* **6**, 993-998.

65. Wells, D., Hoffman, D., and Kedes, L. (1987) *Nucleic Acids Res.* **15**, 2871-2889.

66. Searle, P. F., Davison, B. L., Stuart, G. W., Wilkie, T. M., G., N., and Palmiter, R. D. (1984) *Mol. Cell. Biol.* **4**, 1221-1230.

67. Rorsman, F., Bywater, M., Knott, T. J., Scott, J., and Betsholtz, C. (1988) *Mol. Cell. Biol.* **8**, 571-577.

68. Lewis, S. A., and Cowan, N. J. (1986) *Mol. Cell. Biol.* **6**, 1529-1534.

69. Ackerman, S. L., Minden, A. G., Williams, G. T., Bobonis, C., and Yetung, C. Y. (1991) *Proc. Natl Acad. Sci. U. S. A.* **88**, 7523-7527.

70. Gupta, M. P., Gupta, M., R., Z., and Sukhatme, V. P. (1991) *J. Biol. Chem.* **266**, 12813-12816.

71. Thiel, G., Schoch, S., and Petersohn, D. (1994) *J. Biol. Chem.* **269**, 15294-15301.

72. Molnar, G., Crozat, A., and Pardee, A. B. (1994) *Mol. Cell. Biol.* **14**, 5242-5248.

73. Yoon, J. K., and Lau, L. F. (1993)*J. Biol. Chem.* **268**, 9148-9155.

74. Williams, G. T., and Lau, L. F. (1993) *Mol. Cell. Biol.* **13**, 6124-6136.

75. Thiel, G., Schoch, S., and Petersohn, D. (1994) *J. Biol. Chem.* **21**, 15294-15301.

76. Sukhatme, V. P., Kharta, S., Toback, F. G., Taub, R., Hoover, R. G., and Tsai-Morris, C. H. (1987) *Oncogene Res.* **1**, 343-355.

77. Christy, B. A., Lau, L. F., and Nathans, D. (1988) *Proc. Natl Acad. Sci. U. S. A.* **85**, 7857-7861.

78. Varnum, B. C., Lim, R. W., Sukhatme, V. P., and Herschman, H. R. (1989) *Oncogene* **4**, 119-120.

79. Seyfert, V. L., McMahon, S. B., Glenn, W. D., Yellen, A. J., Sukhatme, V. P., Cao, X., and Monroe, J. G. (1990) *Science* **250**, 797-800.

80. Perez-Castillo, A., Pipaon, C., Garcia, I., and Alemany, S. (1993) *J. Biol. Chem.* **268**, 19445-19450.

81. Nguyen, H., Hoffman-Liebermann, B., and Liebermann, D. A. (1993) *Cell* **72**, 197-209.

82. Cole, A. J., Saffen, D. W., Baraban, J. M., and Worley, P. F. (1989) *Nature* **340**, 474-476.

83. Wisden, W., Errington, M. L., Williams, S., Dunnett, S. B., Waters, C., Hitchcock, D., Evan, G., Bliss, T. V. P., and Hunt, S. P. (1990) *Neuron* **4**, 603-614.

84. Nedivi, E., Hevroni, D., Naot, D., Israeli, D., and Citri, Y. (1993) *Nature* **363**, 718-722.

85. Saffen, D. W., Cole, A. J., Worley, P. F., Christy, B. A., Ryder, K., and Baraban, J. M. (1988) *Proc. Natl Acad. Sci. U. S. A.* **85**, 7795-7799.

86. Dumam, R. S., Craig, J. S., Winston, S. M., Deutch, A. Y., and Hernandez, T. D. (1992) *J. Neurochem.* **59**, 1753-1760.

87. Davis, I. J., Hazel, T. G., and Lau, L. F. (1991) *Molec. Endocrinol.* **5**, 854-859.

88. Watson, M. A., and Milbrandt, J. (1989) *Mol. Cell. Biol.* **9**, 4213-4219.

89. Wilson, T. E., Fahrner, T. J., Johnston, M., and Jeffrey, M. (1991) *Science* **252**, 1296-1300.

90. Chaplin, D. D., Galbraith, L. J., Seidman, J. G., White, P. C., and Parker, K. L. (1986) *Proc. Natl Acad. Sci. U. S. A.* **83**, 9601.

91. Morohashi, K. I., Sogawa, K., Omura, T., and Fujii-Kuriyama, Y. (1987) *J. Biochem.* **101**, 879.

92. Shibahara, S.*et al.* (1983) *EMBO J.* **2**, 775.

93. Sweetser, D. A. *et al.* (1987) *J. Biol. Chem.* **262**, 16060.

94. Cook, J. S. *et al.* (1988) *Proc. Natl Acad. Sci. U. S. A.* **85**, 2949.

95. Protter, A. A. *et al.* (1984) *DNA* **3**, 449.

96. Blackhart, B. D. *et al.* (1986) *J. Biol. Chem* **261**, 15364.

97. Demmer, L. A. (1987) *J. Biol. Chem* **262**, 2458.

98. Sasaki, H., Yosioka, N., Takagi, Y., and Sakaki, Y. (1985) *Gene* **37**, 191.

99. Osumi, T., Ishii, N., Miyazawa, S., and Hashimoto, T. (1987) *J. Biol.Chem.* **262**, 8138.

100. Evans, B. A., and Richards, R. I. (1985) *EMBO J.* **4**, 133.

101. Crowder, C. M., and Merlie, J. P. (1988) *Mol. Cell. Biol.* **8**, 5257.

102. DePinho, R. A., and al., e. (1986) *Proc. Natl. Acad. Sci U. S. A.***83**, 1827.

103. Wilson, T. E., Mouw, A. R., Weaver, C. A., Milbrandt, J., and Parker, K. L. (1993) *Mol. Cell. Biol.* **13**, 861-868.

104. Woronicz, J. D., Calnan, B., Ngo, V., and Winoto, A. (1994) *Nature* **367**, 277-280.

105. Liu, Z.-G., Smith, S. W., McLaughlin, K. A., Schwartz, l. M., and Osborne, B. A. (1994) *Nature* **367**, 281-284.

106. Machida, C. M., Rodland, K. D., Matrisian, L., Magun, B. E., and Ciment, G. (1989) *Neuron* **2**, 1587-1596.

107. Hengerer, B., Lindholm, D., Heumann, R., Ruther, U., Wagner, E. R., and Thoenen, H. (1990) *Proc. Natl. Acad. Sci U. S. A.* **87**, 3899-3903.

108. Gizang-Ginsberg, E., and Zipp, E. B. (1990) *Genes & Dev.* **4**, 477-491.

109. Ginty, D. D., Bading, H., and Greenberg, M. E. (1992) *Curr. Opin. Neurobiol.* **2**, 312-316.

110. Goelet, P., Castellucci, V. F., Schacher, S., and Kandel, E. R. (1986) *Nature* **322**, 419-422.

111. Black, I. B., Adler, J. E., Dreyfus, C. F., Friedman, W. F., LaGamma, E. F., and Roach, A. H. (1987) *Science* **236**, 1263-1268.

112. Comb, M., Hyman, S. E., and Goodman, H. M. (1987) *Trends Neurosci.* **10**, 473-478.

113. Hai, C. M., and Murphy, R. A. (1989) *Annu. Rev. Physiol.* **51**, 285-298.

114. Malenka, R. C. (1994) *Cell* **78**, 535-538.

115. Kandel, E. (1981) *Nature* **293**, 697-700.

116. Smith, S. J., and Augustin, G. J. (1988) *Trends Neurosci.* **11**, 458-464.

117. Katz, B., and Miledi, R. (1967) *J. Physiol. (London)* **189**, 535-544.

118. Strynadka, G., and James, H. (1989) *Annu. Rev. Biochem.* **58**, 951-998.

119. Bredt, D. S., and Snyder, S. H. (1990) *Proc. Natl. Acad. Sci U. S. A.* **87**, 682-685.

120. Forstermann, U., Pollock, J. S., Schmidt, H. H. W., Heller, M., and Murad, F. (1991) *Proc. Natl. Acad. Sci U. S. A.* **88**, 1788-1792.

121. Dawson, T. M., Dawson, V. L., and Snyder, S. H. (1992) *Annals Neurol.* **32**, 297-311.

122. Choi, E. J., Xia, Z., Villacres, E. C., and Storm, D. R. (1993) *Curr. Opin. Cell Biol.* **5**, 269-273.

123. Xu, A., Hawkins, C., and Narayanan, N. (1993) *J. Biol.Chem.* **268**, 8394-8397.

124. Ryu, S. H., Lee, S. Y., and Rhee, S. G. (1987) *FASEB J.* **1**, 388-393.

125. Picciotto, m. R., Czernik, A. J., and Nairn, A. C. (1993) *J. Biol. Chem.* **268**, 26512-26521.

126. Nairn, A. C., Bhagat, B., and Palfrey, H. C. (1985) *Proc. Natl. Acad. Sci U. S. A.* **82**, 7939-7943.

127. Nairn, A. C., and Palfrey, H. C. (1987) *J. Biol.Chem.* **262**, 17299-17303.

128. Ohmstede, C.-A., Bland, M. M., Merrill, B. M., and Sahyoun, N. (1991) *Proc.Natil Acad. Sci.U. S. A* . **88**, 5784-5788.

129. Means, A. R., Cruzalegui, F., Le Magueresse, b., Needleman, D. S., Slaughter, G. R., and Ono, T. (1991) *Mol. Cell. Biol.* **11**, 3960-3971.

130. Sikela, J. M., Law, M. L., Kao, F. T., Hartz, J. A., Wei, Q., and Hahn, W. (1989) *Genomics* **4**, 21-27.

131. Mazzanti, M., DeFelice, L. J., Cohen, J., and Malter, H. (1990) *Nature* **343**, 764-767.

132. Lipscombe, D., Madison, D. V., Poenie, M., Reuter, H., Tsien, R. Y., and Tsien, R. W. (1988) *Proc. Natl. Acad. Sci U. S. A.* **85**, 2398-2404.

133. Williams, D. A., Fogarty, K. E., Tsien, R. Y., and Fay, F. S. (1985) *Nature* **318**, 558-561.

134. Hernandez-Cruz, A., Sala, F., and Adams, P. R. (1990) *Science* **247**, 858-862.

135. Przywara, D. A., Bhave, S. V., Bhave, A., Wakade, T. D., and Wakade, A. R. (1991) *FASEB J.* **5**, 217-222.

136. Himpens, B., de Smedt, H., Droogmans, G., and Casteels, R. (1992) *Am. J. Physiol.* **263**, C95-C105.

137. Nicotera, P., Orrenius, S., Nilsson, T., and Berggren, P. O. (1990) *Proc. Natl. Acad. Sci U. S. A.* **87**, 6858-6862.

138. Matter, N., Ritz, M., Freyermuth, S., Rogue, P., and Malviya, A. N. (1993) *J. Biol. Chem.* **268**, 732-736.

139. Means, A. R. (1994) *FEBS Lett.* **347**, 1-4.

140. Harper, J. F., Cheung, W. Y., Wallace, R. W., Levine, S. N., and Steiner, A. L. (1980). Calcium and Cell Function. W. W. Y. (Eds.), Calcium and Cell Function (pp. 273-290). New York : Academic Press.

141. Serratosa, J., M.J., P., Bachs, M. J., and Carafoli, E. (1988) *Biochem. Biophys. Res. Comm.* **150**, 1162-1169.

142. Mata, M., and Fink, D. J. (1988) *Brain Res.* **475**, 297-304.

143. Pujol, M. J., Bosser, R., Vendrell, M., Serratosa, J., and Bachs, O. (1993) *J. Neurochem.* **60**, 1422-1428.

144. Bosser, r., Aligue, r., Guerini, D., Agell, N., carafoli, E., and Bachs, O. (1993) *J. Biol. Chem.* **268**, 15477-15483.

145. Bachs, O., Agell, N., and Carafoli, E. (1992) *Biochimica et Biophysica Acta* **1113**, 259-270.

146. Gilchrist, J. S. C., Czubryt, M. P., and Pierce, G. N. (1994) *Mol. Cell. Biochem.* **135**, 79-88.

147. White, B. E. (1985) *J. Biol. Chem.* **260**, 1213-1217.

148. Day, R. N., and Maurer, R. A. (1990) *Mol. Endocrinol.* **4**, 736-742.

149. Delidow, b. C., Lail-Trecker, M., and White, B. A. (1992) *Mol. Endocrinol.* **6**, 1268-1276.

150. Loeffler, J. P., Kley, N., Louis, J. C., and Demeneix, B. A. (1989) *J. Neurochem.* **52**, 1279-1283.

151. German, M. S., Moss, L. G., and Rutter, W. J. (1990) *J. Biol. Chem.* **265**, 22063-22066.

152. Kilbourne, E. J., Nankova, B. B., Lewis, E. J., McMahon, A., Osaka, H., Sabban, D. B., and Sabban, E. L. (1992) *J. Biol. Chem.* **267**, 7563-7569.

153. Brown, J. h., and Trejo, J. (1991) *J. Biol. Chem.* **266**, 7876-7882.

154. Walke, W., Staple, J., Adams, L., Gnegy, M., Chahine, K., and Goldman, D. (1994) *J. Biol. Chem.* **269**, 19447-19456.

155. Sheng, M., Dougan, S. T., McFadden, G., and Greenberg, M. E. (1988) *Mol. Cell. Biol.* **8**, 2787-2796.

156. Sheng, M., McFasdden, G., and Greenberg, M. E. (1990) *Neuron* **4**, 571-582.

157. Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991) *Science* **252**, 1427-1430.

158. Bading, H., Ginty, D. D., and Greenberg, M. E. (1993) *Science* **260**, 181-186.

159. Duchemin, A. M., Enyeart, J. A., Biagi, B. A., Foster, D. N. F., Mlinar, B., and Enyeart, J. J. (1992) *Mol. Endocrinol.* **6**, 563-571.

160. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) *Proc. Natl. Acad. Sci U. S. A.* **83**, 6682-6686.

161. Goodman, R. H. (1990) *Ann. Rev. Neurosci.* **13**, 111-127.

162. Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P. L., and Verma, I. M. (1988) *Genes and Dev.* **2**, 1529-1538.

163. Gonzalez, G. A., and Montminy, M. R. (1989) *Cell* **59**, 675-680.

164. Dash (1991) *Proc. Natl. Acad. Sci U. S. A.***88**, 5061-5065.

165. Schwaninger, M., Lux, G., Blume, R., Oetjen, E., Hidaka, H., and Knepel, W. (1993) *J. Biol. Chem.* **268**, 5168-5177.

166. Liu, F., Thompson, M. A., Wagner, S., Greenberg, M. E., and Green, M. R. (1993) *J. Biol. Chem.* **268**, 6714-6720.

167. Griffith, L. C., and Schulman, H. (1988) *J. Biol. Chem.* **263**, 9542-9549.

168. MacNicol, M., Jefferson, A. B., and Schulman, H. (1990) *J. Biol. Chem.* **265**, 18055-18058.

169. Kapillof, M. S., Matthis, J. M., Nelson, C. A., Lin, C. R., and Rosenfeld, M. G. (1991) *Proc. Natl. Acad. Sci U. S. A.* **88**, 3710-3714.

170. Chang, C. J., Chen, T. T., Lei, D. S., Chen, D. S., and Lee, S. C. (1990) *Mol. Cell. Biol.* **10**, 6642-6653.

171. Descombe, P., Chojkier, M., Lichtsteiner, E., Falvey, E., and Schibler, U. (1990) *Genes & Dev.* **3**, 1541-1551.

172. Poli, V., Mancini, F. P., and Cortese, R. (1990) *Cell* **63**, 643-653.

173. Metz, R., and Ziff, E. (1991) *Oncogene* **6**, 2165-2178.

174. Wegner, M., Cao, Z., and Rosenfeld, M. G. (1992) *Science* **256**, 370-373.

175. Kahan, B. (1989) *New England J. Med.* **321**, 1725-1738.

176. Kino, T. H., Hatanake, S., Miyata, N., Inamura, M., Nishiyama, T., Yajima, T., Goto, M., Okuhara, M., Kohsaka, H. A., and Ochai, T. (1987) *J. Antibio* **40**, 1256-1265.

177. Fung, J., Todo, S., Tzakis, A., Alessiani, M., Abuelmagd, K., Jain, A., Bronster, O., Martin, M., Gordon, R., and Starzl, T. (1991) *Transplant. Proc.* **23**, 1902-1905.

178. Kronke, M., Leonard, W., Depper, J., Ara, F., Wong-Stall, F., and Gallo, R. (1984) *Proc. Natl. Acad. Sci U. S. A.* **81**, 5214-5218.

179. Granelli-Piperno, A., Andrus, L., and Steinman, R. M. (1986) *J. Exp. Med.* **163**, 922-937.

180. Tocci, M. J., Matkocich, D. A., Collier, K. A., Kwok, P., Dumont, F., Lin, S., Kegudicibus, S., Siekierka, J., Chin, J., and Hutchinson, N. I. (1990) *J. Immunol.* **143**, 718-726.

181. Handschumacher, R., Harding, M., Rice, J., Druggs, R., and Speicher, D. (1984) *Science* **226**, 544-547.

182. Siekierka, J., Hung, S., Poe, M., Lin, C., and Sigal, N. (1989) *Nature* **341**, 755-757.

183. Harding, M., Galat, A., Uehling, D., and Schreiber, S. (1989) *Nature* **341**, 758-760.

184. Friedman, J., and Weissman, I. (1991) *Cell* **66**, 799-806.

185. Liu, J., Farmer, J. D. J., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) *Cell* **66**, 807-815.

186. Dumont, F., Staruch, M., Koprak, S., Siekierka, J., Lin, C., Harrison, R., Sewell, T., Kindt, T., Beattie, T., Wyvratt, M., and Sigal, N. (1992) *J. Exp. Med.* **176**, 751-760.

187. Sigal, N., Dumont, F., Durette, P., Siekierka, J., Peterson, L., Rich, D., Dunlap, B., Staruch, M., Melino, M., Koprak, S., Williams, D., Witzel, B., and Pisano, J. (1991) *J. Exp. Med.* **173**, 619-628.

188. Fruman, D., Klee, C., Bierer, B., and Burakoff, S. (1992) *Proc. Natl. Acad. Sci U. S. A.* **89**, 3686-3690.

189. Kaye, R., Fruman, D., Bierer, B., Albers, M., Zydowsky, L., Ho, S., Jin, Y., Castells, M., S., S., Walsh, C., Burakoff, S., Austen, K., and Katz, H. (1992) *Proc. Natl. Acad. Sci U. S. A.* **89**, 8542-8546.

190. O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., and O'Neill, E. A. (1992) *Nature* **357**, 692-694.

191. Clipstone, N. A., and Crabtree, G. R. (1992) *Nature* **357**, 695-697.

192. Tokumitsu, H., Masuda, E. S., Tsuboi, A., Arai, K., and Arai, N. (1993) *Biochem. Biophys. Res. Comm.* **196**, 737-744.

193. McCaffrey, P., Perrino, B. A., Soderling, T. R., and Rao, A. (1993) *J. Biol Chem.* **268**, 3747-3752.

194. Jain, J., McCaffrey, P. G., Miner, Z., Kerppola, T. K., Lambert, J. N., Verdine, G. L., Curran, T., and Rao, A. (1993) *Nature* **365**, 352-355.

195. Flanagan, W. M., Corthésy, B., Bram, R. J., and Crabtree, G. R. (1991) *Nature* **352**, 803-807.

196. Schwaninger, M., Blume, R., Oetjen, E., Lux, G., and Knepel, W. (1993) *J. Biol. Chem.* **268**, 23111-23115.

197. Fukunaga, K., Stoppini, L., Miyamoto, E., and Muller, D. (1993) *J. Biol. Chem.* **268**, 7863-7867.

198. Schönthal, A., Sugarman, J., Heller Brown, J., Hanley, M. R., and Feramisco, J. R. (1991) *Proc. Natl. Acad. Sci U. S. A.* **88**, 7096-7100.

199. Collart, M. A., Tourkine, N., Belin, D., Vassalli, P., Jeanteur, P., and Blanchard, J.-M. (1991) *Mol. Cell. Biol.* **11**, 2826-2831.

200. Mechti, N., Piechaczyk, M., Blanchard, J.-M., Jeanteur, P., and Lebleu, B. (1991) *Mol. Cell. Biol.* **11**, 2832-2841.

201. Werlen, G., Belin, D., Conne, B., Roche, E., Lew, D. P., and Prentki, M. (1993) *J. Biol. Chem.* **268**, 16596-16601.

202. Velcich, A., and Ziff, E. B. (1990) *Mol. Cell. Biol.* **10**, 6273-6282.

203. Berkowitz, L. A., Riabowol, K. T., and Gilman, M. Z. (1989) *Mol. Cell. Biol.* **9**, 4272-4281.

204. Schontal, A., Buscher, M., Angel, P., Rahmsdorf, H. J., Ponta, J., Hattori, K., Chiu, R., Karin, M., and Herrlich, P. (1989) *Oncogene* **4**, 629-636.

205. Shaw, P. E., Frasch, S., and Nordheim, A. (1989) *EMBO J.* **8**, 2567-2574.

206. Kadonaga, J. T., Carner, K. R., Maziarz, F. R., and Tjian, R. (1987) *Cell* **51**, 1079-1090.

207. Rauscher, F. J., III, Morris, J. F., Tournay, O. E., Cook, D. M., and Curran, T. (1990) *Science* **250**, 1259-1262.

208. Dynan, W. S., and Tjian, R. (1983) *Cell* **35**, 79-87.

209. Changelian, P. S., Feng, P., King, T. C., and Milbrandt, J. (1989) *J. Biol. Chem.* **86**, 377-381.

210. DeFranco, C., Damon, D. H., Endoh, M., and Wagner, J. A. (1993) *Mol. Endocrinol.* **7**, 365-378.

211. Ginty, D. D., Glowacka, D., Bader, D. S., Hidaka, H., and Wagner, J. A. (1991) *J. Biol. Chem.* **266**, 17454-17458.

212. Peunova, N., and Enikolopov, G. (1993) *Nature* **364**, 450-453.

213. Aszodi (1991) *Proc. Natl. Acad. Sci U. S. A.* **88**, 5832-5838.

214. Hirai, S., Kawasaki, H., Yaniv, M., and Suzuki, K. (1991) *FEBS Lett.* **287**, 57-61.

215. Carillo, S., Pariat, M., Steff, A. M., Roux, P., Etienne-Julan, M., Lorca, T., and Piechaczyk, M. (1994) *Oncogene* **9**, 1679-1689.

216. Hunter, T., and Karin, M. (1992) *Cell* **70**, 375-387.

217. Nasmith, K., Adolf, G., Lydall, D., and Seddon, A. (1990) *Cell* **62**, 631-647.

218. Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmith, K. (1991) *Cell* **66**, 743-758.

219. Levy, D. E., Kessler, D. S., Pine, R., and Darnell, J. E. (1989) *Genes & Dev.* **3**, 1362-1371.

220. Kessler, D. S., Veals, S. A., Fu, X. Y., and Levy, D. E. (1990) *Genes & Dev.* **4**, 1753-1765.

221. Gilmore, T. D. (1991) *Trends Genet.* **7**, 1-5.

222. Schmitz, J. L., Henkel, T., and Baeuerle, P. A. (1991) *Trends Cell Biol* **1**, 130-137.

223. Baeuerle, P. A., and Baltimore, D. (1988) *Cell* **53**, 211-217.

224. Baeuerle, P. A., and Baltimore, D. (1988) *Science* **242**, 540-546.

225. Shirakawa, F., and Mizel, S. B. (1989) *Mol. Cell. Biol.* **9**, 2424-2430.

226. Gosh, S., and Baltimore, D. (1990) *Nature* **344**, 678-682.

227. Kerr, L. D., Inoue, J., Davis, N., Link, E., Baeuerle, P. A., Bose, H. R. J., and Verma, I. M. (1991) *Genes & Dev.* **5**, 1464-1476.

228. Link, E., Kerr, L. D., Schreck, R., Zabel, U., Verma, I. M., and Baeuerle, P. A. (1992) *J. Biol. Chem.* **267**, 239-246.

229. Luscher, B., Christenson, E., Litchfield, D. W., Krebs, E. G., and Eisenman, R. N. (1990) *Nature* **344**, 517-522.

230. Alvarez, E., Northwood, I. C., Gonzalez, S. A., Latour, D. A., Seth, A., Abate, C., Curran, T., and Davis, R. J. (1991) *J. Biol. Chem.* **266**, 15277-15285.

231. Lin, A., Frost, J., Deng, T., Smeal, T., al-Alawi, N., Kikkawa, U., Hunter, T., Brenner, D., and Karin, M. (1992) *Cell* **70**, 777-789.

232. Boyle, W. J., Van Der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **99**, 110-149.

233. Smeal, T., Binetruy, B., Mercola, D., Birrer, M., and Karin, M. (1991) *Nature* **354**, 494-496.

234. Smeal, T., Binetruy, B., Mercola, D., Heidecker, G., Rapp, U. R., and Karin, M. (1991) *Mol. Cell. Biol.* **12**, 3507-3513.

235. Berberich, S. J., and Cole, M. D. (1992) *Genes & Dev.* **6**, 166-176.

236. Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P., and Olson, E. N. (1992) *Cell* **71**, 1181-1194.

237. Kapillof, M. F., Farkash, Y., Wegner, M., and Rosenfeld, M. G. (1991) *Science* **253**, 786-789.

238. Segil, N., Roberts, S. B., and Heintz, N. (1991) *Science* **254**, 1814-1816.

239. Manak, J. R., de Bisschop, N., Kris, R. M., and Prywes, R. (1990) *Genes & Dev.* **4**, 955-967.

240. Manak, J. R., and Prywes, R. (1991) *Mol. Cell. Biol.* **11**, 3652-3659.

241. Janknecht, R., Hipskind, R. A., Houthaeve, T., Nordheim, A., and Stunnenberg, H. G. (1992) *EMBO J.* **11**, 1045-1054.

242. Marais, R. M., Hsuan, J. J., McGuigan, C., Wynne, J., and Treisman, R. (1992) *EMBO J.* **11**, 97-105.

243. Sommercorn, J., Mulligan, J. A., Lozeman, F., and Krebs, E. G. (1989) *Proc. Natl. Acad. Sci U. S. A.* **84**, 8834-8838.

244. Carrol, D., and Marshak, D. R. (1989) *J. Biol. Chem.* **264**, 7345-7348.

245. Montminy, M. R., and Bilezikjian, L. M. (1987) *Nature* **328**, 175-178.

246. Hoeffler, J. P., Meyer, T. E., Yun, Y., Jameson, J. L., and Habener, J. F. (1988) *Science* **242**, 1430-1433.

247. Gonzalez, G. A., Yamamoto, K. K., Fischer, W. H., Karr, D., Menzel, P., Biggs, I. w., Vale, W. W., and Montminy, M. R. (1989) *Nature* **337**, 749-752.

248. Lalli, E., and Sassone-Corsi, P. (1994) *J. Biol. Chem.* **269**, 17359-17362.

249. Meyer, T. E., and Habener, J. F. (1993) *Endocrine Reviews* **14**, 269-290.

250. Laoide, B. M., Foulkes, N. S., Schlotter, F., and Sassone-Corsi, P. (1993) *EMBO J.* **12**, 1179-1191.

251. Molina, C. A., Foulkes, N. S., Lalli, E., and Sassone-Corsi, P. (1993) *Cell* **75**, 875-886.

252. Cowell, I. G., Skinner, A., and Hurst, H. C. (1992) *Mol. Cell. Biol.* **12**, 3070-3077.

253. Karpinski, B. A., Morle, G. D., Huggenvik, J., Uhler, M. D., and Leiden, J. M. (1992) *Proc. Natl. Acad. Sci U. S. A.* **89**, 4820-4824.

254. Hoeffler, J. P., Meyer, T. E., G.W., W., and Habener, J. F. (1990) *Mol. Endocrinol.* **4**, 920-930.

255. Yamamoto, K. K., Gonzalez, G. A., Menzel, P., Rivier, J., and Montminy, M. R. (1990) *Cell* **60**, 611-617.

256. Berkowitz, L. A., and Gilman, M. Z. (1990) *Proc. Natl. Acad. Sci U. S. A.* **87**, 5258-5262.

257. Ruppert, S., Cole, T. J., Boshart, M., Schmid, E., and Schutz, G. (1992) *EMBO J.* **11**, 1503-1512.

258. Van Hog, M., Leuther, K. K., Kodalek, T., and Johnson, S. A. (1993) *Cell* **72**, 587-594.

259. Roach, P. J. (1991) *J. Biol. Chem.* **266**, 14139-14142.

260. Gonzalez, G. A., Menzel, P., Leonard, J., Fisher, W. H., and Montminy, M. R. (1991) *Mol. Cell. Biol.* **11**, 1306-1312.

261. Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H. I., and Montminy, M. R. (1988) *Nature* **334**, 494-498.

262. Lee, C. Q., Yun, Y., Hoeffler, J. P., and Habener, J. F. (1990) *EMBO J.* **9**, 4455-4465.

263. Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., and Montminy, M. (1992) *Cell* **70**, 105-113.

264. Alberts, A. L., Montminy, M., Shenolikar, S., and Feramisco, J. R. (1994) *Mol. Cell. Biol.* **14**, 4398-4407.

265. Wadzinski, B. E., Wheat, W. H., Jaspers, S., Peruski Jr., L. F., Lickteig, R. L., Johnson, G. L., and Klemm, D. J. (1993) *Mol. Cell. Biol.* **13**, 2822-2834.

266. Wheat, W. H., Roesler, W. J., and Klemm, D. J. (1994) *Mol. Cell. Biol.* **14**, 5881-5890.

267. Wadzinski, B. E., Wheat, W. H., Jaspers, S., Peruski Jr., L. F., Lickteig, R. L., Johnson, G. L., and Klemm, D. J. (1993) *Mol. Cell. Biol.* **13**, 2822-2834.

268. Scheidtmann, K. H., Mumby, M. C., Rundell, K., and Walter, G. (1991) *Mol. Cell. Biol.* **11**, 1996-2003.

269. Yang, S. R., Lickteig, L. R., Estes, R., Rundell, K., walter, G., and Mumby, M. C. (1991) *Mol. Cell. Biol.* **11**, 1988-1995.

270. Sontag, E., fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M. (1993) *Cell* **75**, 887-897.

271. Chrivia, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature* **365**, 855-859.

272. Kwok, R. P. S., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G. E., Green, M. R., and Goodman, R. H. (1994) *Nature* **370**, 223-226.

273. Carey, M. (1994) *Nature* **368**, 402-403.

274. Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. (1994) *Nature* **370**, 226-229.

275. Seth, A., Alvarez, E., Gupta, S., and Davis, R. J. (1991) *J. Biol. Chem.* **266**, 23521-23524.

276. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) *Nature* **353**, 670-674.

277. Binetruy, B., Smeal, T., and Karin, M. (1991) *Nature* **351**, 122-127.

278. Smeal, T., Binetruy, B., Mercola, D., Heidecker, G., Rapp, U. R., and Karin, M. (1992) *Mol. Cell. Biol.* **12**, 3507-3513.

279. Kamm, K. E., and Stull, J. T. (1985) *J. Biol. Chem.* **269**, 9912-9920.

280. Hartshorne, D. J. (1987) *Physiology of the Gastrointestinal Tract* (Johnson L.R. ed.) Raven Press, New-York.

281. Hashimoto, Y., and Soderling, T. R. (1990) *Arch. Biochem. Biophys.* **278**, 41-45.

282. Cohen, P. (1988) *Mol. Aspects Cell. Regul.* **5**, 123-144.

283. Mitsui, K., Brady, M., Palfrey, H. C., and Nairn, A. C. (1993) *J. Biol. Chem.* **268**, 13422-13433.

284. Kennedy, M. B., and Greengard, P. (1981) *Proc. Natl. Acad. Sci U. S. A.* 1293-1297.

285. Nairn, A. C., and Greengard, P. (1987) *J. Biol. Chem.* **262**, 7273-7281.

286. Czernik, A. J., Pang, D. T., and Greengard, P. (1987) *Proc. Natl. Acad. Sci U. S. A.* **84**, 7518-7522.

287. Sudhof, T. C., Czernik, A. J., Kao, H. T., Takei, K., Johnston, P. A., Horiuchi, A., Kanazir, S. D., Wagner, M. A., Perin, M. S., De Camilli, P., and Greengard, P. (1989) *Science* **245**, 1474-1480.

288. Picciotto, M. R., Cohn, J. A., Bertuzzi, G., Greengard, P., and Nairn, C. A. (1992) *J. Biol. Chem.* **267**, 18.

289. Nairn, A. C., Picciotto, M. R., Elliott, J. I., and Czernick, A. J. (1994) *FASEB J.* **8**, A1390, Abst. 764.

290. DeRemer, M. F., Saeli, R. J., and Edelman, A. M. (1992) *J. Biol. Chem.* **267**, 13460-13465.

291. DeRemer, M. F., Saeli, R. J., Brautigan, D. L., and Edelman, A. M. (1992) *J. Biol. Chem.* **267**, 13466-13471.

292. Yokokura, H., Picciotto, M. R., Nairn, A. R., and Hidaka, H. (1994) *FASEB J.* **8**, A1389, Abst. 760.

293. Soderling, T. R. (1993) *Biotechnol. Appl. Biochem.* **18**, 185-200.

294. Soderling, T. R., Fukunaga, K., Brickey, D. A., Fong, Y. L., Rich, D. P., Smith, K., and Colbran, R. J. (1991) *Prog. Brain Res.* **89**, 169-183.

295. McGuiness, T. L., Lai, Y., Greengard, P., Woodgett, J. R., and Cohen, P. (1983) *FEBS Lett.* **163**, 329-334.

296. Schulman, H., Kuret, J., Jefferson, A. B., Nose, P., and Spitzer, K. H. (1985) *Biochemistry* **24**, 5320-5327.

297. Schworer, C. M., McClure, R. W., and Soderling, T. R. (1985) *Arch. Biochem. Biophys.* **242**, 137-145.

298. Shenolikar, S., Lickteig, R., Hardie, D. G., Soderling, T. R., Hanley, R. M., and Kelly, P. T. (1986) *Eur. J. Biochem.* **161**, 739-747.

299. Woodgett, J. R., Cohen, P., Yamauchi, T., and Fujisawa, H. (1984) *FEBS Lett.* **170**, 49-54.

300. Yamauchi, T., and Fujisawa, H. (1986) *Biochem. Biophys. Acta* **886**, 57-63.

301. Bennett, M. K., Erondu, N. E., and Kenedy, M. B. (1983) *J. Biol. Chem.* **258**, 12735-12744.

302. Goldenring, J. R., McGuire, J. S., and DeLorenzo, R. J. (1984) *J. Neurochem.* **42**, 1077-1084.

303. Kelly, P. T., McGuiness, T. L., and Greengard, P. (1984) *Proc. Natl. Acad. Sci U. S. A.* **81**, 945-949.

304. Kennedy, M. B., Bennett, M. K., and Erondu, N. E. (1983) *Proc. Natl. Acad. Sci U. S. A.* **80**, 7357-7361.

305. Miller, S. G., and Kennedy, M. B. (1985) *J. Biol. Chem.* **260**, 9039-9046.

306. Bennett, M. K., and Kennedy, M. B. (1987) *Proc. Natl. Acad. Sci U. S. A.* **84**, 1794-1798.

307. Bulleit, R. F., Bennett, M. K., Molloy, S. S., Hurley, J. B., and Kennedy, M. B. (1988) *Neuron* **1**, 63-72.

308. Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., and Burgin, K. E. (1987) *Science* **237**, 293-297.

309. Lin, C. R., Kapillof, M. S., Durgerian, S., Tatemoto, K. and Russo, A. F. (1987) *Proc. Natl. Acad. Sci U. S. A.* **84**, 5962-5966.

310. Tobimatsu, T., Kameshita, I., and Fujisawa, H. (1988) *J. Biol. Chem.* **263**, 16082-16086.

311. Tobimatsu, T., and Fujisawa, H. (1989) *J. Biol. Chem.* **264**, 17907-17912.

312. Nghiem, P., Saati, S. M., Martens, C. L., Gardner, P., and Schulman, H. (1993) *J. Biol. Chem.* **268**, 5471-5479.

313. Schworer, C. M., Rothblum, L. I., Thekkumkara, T. J., and Singer, H. A. (1993) *J. Biol. Chem.* **268**, 14443-14449.

314. Edman, C. F., and Schulman, H. (1994) *Biochem. Biophys. Act.* **1221**, 89-101.

315. Mayer, P. M., Mohlig, M., Schatz, H., and Pfeiffer, A. (1993) *FEBS Lett.* **333**, 315-318.

316. Waalas, S. I., Nairn, A. C., and Greengard, P. (1983) *J. Neurosci.* **3**, 291-301.

317. Waalas, S. I., Nairn, A. C., and Greengard, P. (1983) *J. Neurosci.* **3**, 302-311.

318. Rostas, J. A. P. (1991). Neural and Behavioral Plasticity. The use of domestic Chicken as a Model. (pp. 177-211) R. J. Andrew (Eds.), Oxford : Oxford Science.

319. Corbin, J. D., Sugden, P. H., West, L., Flockhardt, D. A., Lincoln, T. N., and McCarthy, D. (1978) *J. Biol. Chem.* **253**, 3997-4003.

320. Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1986) *J. Biol. Chem.* **261**, 8581-8584.

321. Lai, Y., Nairn, A. C., and Greengard, P. (1986) *Proc. Natl. Acad. Sci U. S. A.* **83**, 4253-4257.

322. Saitoh, Y., Yamamoto, H., Fukunaga, K., Matsukado, Y., and Miyamoto, E. (1987) *J. Neurochem.* **49**, 1286-1292.

323. Miller, S. G., Patton, B. L., and Kennedy, M. B. (1988) *Neuron* **1**, 593-604.

324. Lou, L. L., and Schulman, H. (1989) *J, Neurosci.* **9**, 2020-2032.

325. Fukunaga, K., Kobayashi, T., Tamura, S., and Miyamoto, E. (1993) *J. Biol. Chem.* **268**, 133-137.

326. Mukherji, S., and Soderling, T. R. (1994) *J. Biol. Chem.* **269**, 13744-13747.

327. Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994) *Neuron* **12**, 943-956.

328. Payne, M. E., Schworer, C. M., and Soderling, T. (1983) *J. Biol. Chem.* **258**, 2376-2382.

329. Pearson, R. B., Woodgett, J. R., Cohen, P., and Kemp, B. E. (1985) *J. Biol. Chem.* **260**, 14471-14476.

330. Soderling, T. R., Schworer, C. M., Payne, M. E., Jett, M. E., Porter, D. K., Atkinson, J. L., and Richtand, N. M. (1986). Hormones and cell regulation (pp. 141-157). : J. Nunez, J.E. Dumont and R.J.B. King, eds.

331. Miller, S. G., Patton, B. L., and Kennedy, M. B. (1988) *Neuron* **1**, 593-604.

332. Patton, B. L., Miller, S. G., and Kennedy, M. B. (1988). Soc. Neurosci. Abstr., **14** (pp. 107).

333. Haystead, T. A., Campbell, D. G., and Hardie, D. G. (1988) *Eur. J. Biochem.* **175**, 347-354.

334. Soderling, T. R., Schworer, C. M., El-Maghradi, M. R., and Piljis, S. J. (1986). *Biochem. Biophys. Res. Commun.* **139** 1017-1023.

335. Ando, S., Tokui, T., Yamauchi, T., Sugiura, H., Tanabe, K., and Inagaki, M. (1991) *Biochem. Biophys. Res. Comm.* **175**, 955-962.

336. Malinow, R., Schulman, H., and Tsien, R. W. (1989) *Science* **245**, 862-866.

337. Baitinger, C., Alberton, J., Poenie, M., Schulman, H., and Steinhardt, R. H. (1990) *J. Cell. Biol.* **111**, 1763-1773.

338. Nichols, R. A., Sirha, T. S., Czernik, A. J., Nairn, A. C., and Greengard, P. (1990) *Nature* **343**, 647-651.

339. Lisman, J. (1989) *Proc. Natl. Acad. Sci U. S. A.* **86**, 9574-9578.

340. Soderling, T. R. (1993) *Mol. Cell. Biochem.* **127/128**, 93-101.

341. McGlacde-McCulloh, E., Yamamoto, H., Tan, S.-E., Brickey, D. A., and Soderling, T. R. (1993) *Nature* **362**, 640-642.

342. Tan, S. E., Wenthold, R. J., and Soderling, T. R. (1994) *J. Neurosci.* **14**, 1123-1129.

343. Yakel, J., Vissavajjhala, P., Brickey, D., and Soderling, T. R. (submitted) .

344. McCarron, J. G., McGeown, J. G., Reardon, S., Ikebe, M., Fay, F. S., and Walsh Jr, J. V. (1992) *Nature* **357**, 74-77.

345. Planas-Silva, M. D., and Means, A. R. (1992) *EMBO J.* **11**, 507-517.

346. Lorca, T., Cruzalegui, F. H., Fesquet, D., Cavadore, J. C., Mery, J., Means, A. R., and Doree, M. (1993) *Nature* **366**, 270-273.

347. Morrin, N., Abrieu, A., Lorca, T., martin, F., and Doree, M. (1994) *EMBO J.* **13**, 4343-4352.

348. Ohmstede, C.-A., Jensen, K. F., and Sahyoun, N. E. (1989) *J. Biol. Chem.* **264,** 5866-5875.

349. Miyano, O., Kameshita, I., and Fujisawa, H. (1992) *J. Biol. Chem.* **267**, 1198-1203.

350. Frangakis, M. V., Chatila, T., Wood, E. R., and Sahyoun, N. (1991) *J. Biol. Chem.* **266**, 17592-17596.

351. McDonald, O. B., Merrill, B. M., Bland, M. M., Taylor, L. C. E., and Sahyoun, N. (1993) *J. Biol. Chem.* **268**, 10054-10059.

352. Kameshita, I., and Fujisawa, H. (1993) *J.Biochem. (Tokyo)* **113**, 583-590.

353. Okuno, S., and Fujisawa, H. (1993) *J. Biochem. (Tokyo)* **114**, 167-170.

354. Cruzalegui, F. H., and Means, A. R. (1993) *J. Biol. Chem.* **268**, 26171-26178.

355. Hanissian, S. H., Frangakis, M., Bland, M. M., Jawahar, S., and Chatila, T. A. (1993) *J. Biol. Chem.* **268**, 20055-20063.

356. Jensen, K. F., Ohmstede, C.-A., Fisher, R. S., and Sahyoun, N. (1991) *Proc. Natl. Acad. Sci U. S. A.* **88**, 2850-2853.

357. Sahyoun, N., McDonald, O. B., Farrel, F., and Lapetina, E. G. (1991) *Proc. Natl. Acad. Sci U. S. A.* **88**, 2643-2647.

358. Cohen, P., and Cohen, P. T. W. (1989) *J. Biol. Chem.* **264**, 21435-21438.

359. Klee, C. B., Draetta, G. F., and Hubbard, M. J. (1988) *Adv. Enzymol.* **61**, 149-200.

360. Ingebritsen, T. S., Stewart, A. A., and Cohen, P. (1983) *Eur. J. Biochem.* **132,** 297-307.

361. Bialojan, C., and Takai, A. (1988) *Biochem. J.* **256**, 283-290.

362. Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P., and Hardie, D. G. (1989) *Nature* **337**, .

363. Mumby, M. C., and Walters, G. (1993) *Phys. Rev.* **73**, 673-699.

364. Watanabe, Y., Perrino, B. A., Chang, B. H., and Soderling, T. R. (1994) *J. Biol. Chem.* (in press).

365. Kinkaid, R. L., Nightingale, M. S., and Martin, B. M. (1988) *Proc. Natl. Acad. Sci U. S. A.* **85**, 8983-8987.

366. Hashimoto, Y., Perrino, B. A., and Soderling, B. A. (1990) *J. Biol. Chem.* **265**, 1924-1927.

367. Perrino, B. A., Ng, L. Y., and Soderling, T. R. (1994) *J. Biol. Chem.* (submitted).

368. Hubbard, M. J., and Klee, C. B. (1989) *Biochemistry* **28**, 1868-1874.

369. Perrino, B. A., Fong, Y. L., Brickey, D. A., Saitoh, Y., Ushio, Y., Fukunaga, K., Myamoto, E., and Soderling, T. R. (1992) *J. Biol. Chem.* **267**, 15965-15969.

370. Klee, C. B., Draetta, G., and Hubbard, M. J. (1988) *Adv. Enzymol.* **61**, 149-200.

371. Mulkey, R. M., Herron, E. C., and Malenka, R. C. (1993) *Science* **261**, 1051-1055.

372. Murphy, B. J., Rossie, S., De Jongh, K. S., and Catterwall, W. A. (1993) *J. Biol. Chem.* **268**, 27355-27362.

373. Lieberman, D. L., and Mody, I. (1994) *Nature* **369**, 235-239.

374. Liu, J. P., Sim, A. T. R., and Robinson, P. J. (1994) *Science* **265**, 970-973.

375. Ginty, D. D., Bading, H., and Greenberg, M. E. (1992) *Curr. Opin. Neurobiol.* **2**, 312-316.

376. Sheng, M., and Greeenberg, M. E. (1990) *Neuron* **4**, 477-485.

377. Enslen, H., Sun, P., Brickey, D., Soderling, S., Klamo, E., and Soderling, T. R. (1994) *J. Biol. Chem.* **269**, 15520-15527.

378. Moshizuki, H., Ito, T., and Hidaka, H. (1993) *J. Biol. Chem.* **268**, 9143-9147.

379. Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M., and Hidaka, H. (1990) *J. Biol. Chem.* **265**, 4315-4320.

380. Jones, D. A., Glod, J., Wilson-Shaw, D., Hahn, W. E., and Sikela, J. M. (1991) *FEBS Lett.* **289**, 105-109.

381. Matthews, R. P., Guthrie, C. R., Wailes, L. M., Zhao, X., Means, A. R., and McKnight, G. S. (1994) *Mol. Cell. Biol.* **14**, 6101-6116.

382. Laemmli, U. K. (1970) *Nature* **227**, 680-685.

383. Cathala, G., Savouret, J. T., Mendez, B. L., West, B. L., Karin, M., Martial, J. A., and Baxter, J. D. (1983) *DNA* **2**, 329-335.

384. Brown, A. M. C., Jeltsch, J. M., Roberts, M., and Chambon, P. (1984) *Proc. Natl. Acad. Sci U. S. A.* **81**, 6344-6348.

385. Garglio, P., Bellard, M., and Chambon, P. (1981) *Nucl. Ac. Res.* **9**, 2589-2598.

386. Soderling, T. R., Corbin, J. D., and Park, C. R. (1972) *J. Biol. Chem.* **247**, 1822-1829.

387. Corbin, J. D., Soderling, T. R., and Park, C. R. (1973) *J. Biol. Chem.* **248**, 1813-1821.

388. Danielson, P. E., Forss-Peter, M., Brau, A., Calavetta, J., Douglass, R. J., Milner, R. J., and Sutcliffe, J. G. (1988) *DNA* **7**, 261-267.

389. D'arcangelo, G., and Halegoua, S. (1993) *Mol. Cell. Biol.* **13**, 3146-3155.

390. Farber, L. H., Wilson, F. J., and Wolff, D. J. (1987) *J. Neurochem.* **49**, 404-414.

391. Cohen, P. (1989) *Annu. Rev. Biochem.* **58**, 453-508.

392. Tansey, M. G., Word, R. A., Hidaka, H., Singer, H. A., Schworer, C. M., Kamm, K. E., and T. J. Stull (1992) *J. Biol. Chem.* **267**, 12511-12516.

393. Jain, J., McCaffrey, P. G., Valge-Archer, V. E., and A., R. (1992) *Nature* **356**, 801-804.

394. Natazuka, T., Umemiya-Okada, T., Matsui, T., Saida, T., and Nakao, Y. (1993) *Int. J. Cancer* **54**, 348-354.

395. Sikela, J. M., and Hahn, W. E. (1987) *Proc. Natl. Acad. Sci U. S. A.* **84**, 3038-3042.

396. Frangakis, M. V., Ohmstede, C.-A., and Sahyoun, N. (1991) *J. Biol. Chem.* **266**, 11309-11316.

397. Brickey, D. A., Colbran, R. J., Fong, Y.-L., and Soderling, T. R. (1990) *Biochem. Biophys. Res. Comm.* **173**, 578-584.

398. Sun, P. S., Schoderbek, W. E., and Maurer, R. A. (1992) *Mol. Endocrinol.* **6**, 1858-1866.

399. Colbran, R. J., Fong, Y. L., Schworer, C. M., and R., S. T. (1988) *J. Biol. Chem.* **263**, 18145-18151.

400. Maurer, R. A. (1989) *J. Biol. Chem.* **264**, 6870-6873.

401. Gorman, C., Padmanabhan, R., and Howard, B. H. (1983) *Science* **221**, 551-553.

402. de Wet, J. R., Wood, K. V., deLucas, M., Helinski, D. R., and Subramani, S. (1987) *Mol. Cell. Biol.* **7**, 725-737.

403. Enslen, H., and Soderling, T. R. (1994) *J. Biol. Chem.* **269**, 20872-20877.

404. de Groot, R. P., Derua, R., Goris, J., and Sassone-Corsi, P. (1993) *Mol. Endocrinol.* **7**, 1495-1501.

405. Nghiem, P., Ollick, T., Gardner, P., and Schulman, H. (1994) *Nature* **371**, 347-350.

406. Srinivasan, M., Edman, C. F., and Schulman, H. (1994) *J. Cell Biol.* **126**, 839-852.

407. Kubo, M., Kincaid, R. L., Webb, D. R., and Ransom, J. T. (1994) *Int. Immunol.* **6**, 179-188.

408. Kubo, M., Kincaid, R. L., and Ransom, J. T. (1994) *J. Biol.Chem.* **269**, 19441-19446.

409. Morris, S. M., Kepka-Lenhart, D., McGill, R. L., Curthoys, N. P., and Adler, S. (1992) *J. Biol.Chem.* **267**, 13768-13771.

410. Mahajan, P. B., and Thompson, E. A., Jr (1993) *J. Biol. Chem.* **268**, 16693-16698.

411. Tocci, M. J., Matkovich, D. A., Collier, K. A., Kwok, P., Dumont, F., Lin, S., Degudicibus, S., Siekierka, J. J., Chin, J., and Hutchinson, N. I. (1989) *J. Immunol.* **143**, 718-726.

412. Mattila, P. S., Ullman, K. S., Fiering, S., Emmel, E. A., McCutcheon, M., Crabtree, G. R., and Herzenberg, L. A. (1990) *EMBO J.* **9**, 4425-4433.

413. Lee, G., and Gilman, M. (1994) *Mol. Cell. Biol.* **14**, 4579-4587.

414. Rosen, L. B., Ginty, D. D., Weber, M. J., and Greenberg, M. E. (1994) *Neuron* **12**, 1207-1221.

415. Klee, C. B. (1991) *Neurochemical Res.* **16**, 1059-1065.

416. Ezzell, C. (1993) *J. NIH Res.* **5**, 27-29.

417. Hashimoto, Y., and Sopderling, T. R. (1989) *J. Biol. Chem.* **264**, 16524-16529.

418. Fisch, T. M., Prywes, R., and Roeder, R. G. (1987) *Mol. Cell. Biol.* **7**, 3490-3502.

419. Metz, R., and Ziff, E. (1991) *Genes & Dev.* **5**, 1754-1766.

420. Gille, H., Sharrocks, A. D., and Shaw, P. E. (1992) *Nature* **358**, 414-417.

421. Janknecht, R., Ernst, W. H., Pingoud, V., and Nordheim, A. (1993) *EMBO J.* **12**, 5097-5104.

422. Marais, R. M., Wynne, J., and Treisman, R. (1993) *Cell* **73**, 381-393.

423. Rao, V. N., and Reddy, E. S. P. (1993) *Cancer Res.* **53**, 3449-3454.

424. Zinck, R., Hipskind, R. A., Pingoud, V., and Nordheim, A. (1993) *EMBO J.* **12**, 2377-2387.

425. Lisman, J. (1994) *Trends Neurosci.* **17**, 406-412.

426. Worley, P., Bhat, R. V., Baraban, J. M., Erickson, C. A., McNaughton, B. L., and Barnes, C. A. (1993) *J. Neurosci.* **13**, 4776-4786.

427. Thomas, K. L., Laroche, S., M.L., E., Bliss, T. V. P., and Hunt, S. P. (1994) *Neuron* **13**, 737-745.

428. Davis, L., Banker, G. A., and Steward, O. (1987) *Nature* **330**, 477-479.

429. Davis, L., Burger, B., Banker, G. A., and Steward, O. (1989) *J. Neurosci.* **10**, 3056-3068.

430. Yin, J. C. P., Wallach, J. S., Del Vecchio, M., Wilder, E. L., Zhou, H., Quinn, W., G,, and Tully, T. (1994) *Cell* **79**, 49-58.

431. Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A. J. (1994) *Cell* **79**, 59-68.

432. Dash, P. K., Hochner, B., and Kandel, E. R. (1990) *Nature* **345**, 718-721.

433. Frank, D. A., and Greenberg, M. E. (1994) *Cell* **79**, 5-8.

434. Tokumitsu, H., Brickey, D., Glod, D. A., Sikela, J. and Soderling T. R. (1994) *J. Biol. Chem.* (in press).