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# Nuclear calcium: a key regulator of gene expression

Giles E. Hardingham & Hilmar Bading

*MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England*

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**Through the evolution of multicellular organisms, calcium has emerged as the preferred ion for intracellular signalling. It now occupies a pivotal role in many cell types and nowhere is it more important than in neurons, where it mediates both the relaying and long-term storage of information. The latter is a process that enables learning and memory to be formed and requires the activation of gene expression by calcium signals. Evidence from a number of diverse organisms shows that transcription mediated by the transcription factor CREB is critical for learning and memory. Here we review the features of CREB activation by calcium signals in mammalian cells. In contrast to other transcription factors, its regulation is dependent on an elevation of nuclear calcium concentration, potentially placing this spatially distinct pool of calcium as an important mediator of information storage.**

**Keywords:** calcium, CREB, gene expression

## Introduction

Calcium ions in the cell are very important messengers in cell signalling. They are responsible for coupling many of external events or stimuli to the cell's responses to those stimuli. Calcium has a central role to play in the nervous system, as well as mediating other important processes such as activation of the immune system (Cardenas & Heitman 1995), and fertilisation (Shen 1995).

As eukaryotic cells evolved, the calcium ion has been selected as an intracellular second messenger in preference to other monatomic ions prevalent in the cellular environment, namely magnesium, sodium, potassium and chloride ions. The reasons why this is the case are discussed more fully in a review by Carafoli and Penniston (1982) and essentially centre around the need for an intracellular messenger to bind tightly and with high specificity to downstream components of the signalling cascade (often enzymes) and for the capacity for the concentration of the messenger to vary considerably between elevated and basal levels in a manner that is as energetically efficient as possible.

The singly charged ions of sodium, potassium and chlorine would not bind as tightly to the binding site of proteins as doubly charged calcium. In addition, potassium and chloride ions are considerably larger than calcium, meaning even weaker interactions. The doubly charged magnesium ion is smaller than the calcium ion but rather than creating strong interactions with the protein binding site it cannot be effectively co-ordinated by protein binding sites, being too inflexible, the ion ends up forming bonds with water molecules as well as the protein. As the co-ordination number of magnesium is invariant (six) this means less bonds are made with the protein and so fewer bonds need braking to free the magnesium. Thus it seems that the calcium ion strikes a happy balance between strength of interaction with electron donating groups on the protein, and a certain level of flexibility that enables it to interact tightly and specifically with the appropriate protein.

In addition, it is energetically favourable to utilise calcium as a second messenger. Basal levels of free calcium in the cell are necessarily very low (~$10^{-7}$M) as higher levels would combine with phosphate ions in the cell to form a lethal precipitate. The very low basal levels of intracellular calcium compared to

Correspondence should be addressed to: Hilmar Bading, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England. Telephone: ++44–1223–402308;
Fax: ++44–1223–402310; Email: hb1@mrc-lmb.cam.ac.uk

other ions (~$10^{-3}$M for magnesium) make it energetically efficient to use it as a second messenger-a relatively small amount of calcium needs to pass in to the cytoplasm to increase the concentration of the ion several fold and similarly, relatively little energy need be spent pumping it out again to return the concentration to basal levels

The purpose of this review is to show how calcium acts as a second messenger in the mammalian neurons to couple electrical activity to cellular events required for long term adaptive changes in the nervous system. Particular attention will be paid to nuclear calcium signals, recently been shown to be important in the activation of a transcription factor CREB that time and again crops up in studies into learning and memory in a wide variety of organisms

## Calcium as an intracellular second messenger

Many cell types rely on an elevation of intracellular calcium to activate essential biological functions. This elevation can occur via either influx of calcium through proteinaceous channels into the cell from the extracellular medium, or through the release of calcium from internal stores. For example, T-lymphocytes rely on both these mechanisms to trigger their activation in response to antigen presentation to the T-cell receptor (Cardenas & Heitman 1995). Muscle contraction is also dependent on calcium release from internal stores and is mediated by troponin and tropomysin (Eisenberg and Hill 1985). Calcium too plays a key role in the earliest stages of development-the first observable event in fertilisation is a rise in intracellular calcium initiated at the sperm entry point (Shen 1995).

Calcium is also a critical component in the communication between neurons. An electrical signal (action potential) travelling the length of a neuron (call it neuron 'A') will arrive at the axon terminal and trigger calcium entry into the terminal through voltage-dependent calcium channels. This in turn results in calcium-dependent neurotransmitter release into the synaptic cleft-the gap between the axon terminal of neuron A and the dendrite or cell body of neuron B. This neurotransmitter causes an electrical change in neuron B through the activation of neurotransmitter-gated ion channels. Thus, calcium is responsible for coupling action potentials to neurotransmitter release and enabling information to be passed on from neuron to neuron. However, as well as providing the nuts and bolts of neuronal communication, it is responsible for mediating far more subtle, long-lasting functions i.e. learning and memory. Early clues came from the observation that blocking a calcium channel that is gated by the neurotransmitter glutamate resulted in impaired learning and blockade of an *in vitro* model for learning , long-term potentiation (see below) and heightened interest in neuronal calcium signalling (Morris *et al.* 1986).

## The nervous system: an organ of never ending change

An essential characteristic of an animal's nervous system is that it undergoes lasting structural and functional changes in response to the briefest of stimuli (Bliss and Collingridge 1993). This property of activity dependent plasticity is central to the way connections between neurons are forged and strengthened in the developing and mature animal. The mature animal depends on activity-dependent plasticity to change neuronal connectivity and strength in ways that enable the process of learning and memory (Malenka 1994). It is therefore a fundamental goal of neurobiologists to understand how electrical activity results in these long-lasting changes.

### *Memory and long term potentiation in mammals*

Neuronal plasticity can be split into two phases. During the early phase, seconds to minutes after electrical activity, changes in neuronal connections take place via the modification of existing proteins, for example by phosphorylation (Robertson *et al.* 1996). In the later stage (minutes to hours), new gene expression and subsequent protein synthesis converts these initial transient changes into long-lasting ones. In the mammalian brain these changes in gene expression are primarily triggered by calcium influx into neurons and involve the activation of intracellular signalling pathways (Bading *et al.* 1993).

The hippocampus has long been the focus of studies into memory formation in mammals since clinicians observed that patients with hippocampal lesions could not form new memory, suffered anterograde and retrograde amnesia and were deficient in spatial learning tasks (Milner *et al.* 1968, for reviews, see Nadel & Moscovitch 1997; Whishaw *et al.* 1997). The phenomenon of long term potentiation (LTP) is an extensively studied model for learning and memory. LTP is an activity-dependent increase in synaptic efficacy that can last for days to weeks in intact animals (Bliss & Lomo 1973; Bliss & Collingridge

1993). It is induced by repeated, high frequency stimulation of hippocampal neurons. LTP is characterised by an early, protein synthesis independent phase and a late phase whose establishment is blocked by protein synthesis inhibitors (Frey *et al.* 1993) and requires a critical period of transcription after the LTP-inducing stimuli have been applied (Nguyen *et al.* 1994).

The molecular basis of LTP is not fully understood. However, it is thought to involve changes at both the pre-synaptic as well as post-synaptic level. A pre-synaptic modification is thought to be an enhancement of neurotransmitter release, in other words improved information transmission. Post-synaptically, improved information reception is thought to occur, via an enhanced response to neurotransmitter. This occurs through the modification of existing neurotransmitter receptors and/or the production of new/more neurotransmitter receptors (reviewed by Bliss & Collingridge 1993, Larkman & Jack, 1995).

The post-synaptic signalling pathways that lead to LTP have been the subject of much research. Lynch *et al.* (1983) found that intracellular injection of the calcium chelator, EGTA, blocked induction of LTP in the hippocampus. This result implicated the post-synaptic cell, and in particular, calcium signalling, in the induction process. Release of calcium into post-synaptic neurons by photolysis of a caged calcium compound produced a potentiation of synaptic transmission (Malenka *et al.* 1988). It was also established that calcium influx through either the NMDA type of glutamate receptor or L-type voltage sensitive calcium channels can trigger LTP (Morris *et al.* 1986; Bliss & Collingridge 1993; Kullman & Siegelbaum 1995). This calcium influx may be augmented by release from internal calcium stores, for example, calcium store depletion can inhibit the induction of LTP (Harvey and Collingridge 1992). The generation of inositol 1, 4, 5-trisphosphate (InsP3) as a result of the activation of metabotropic glutamate receptors (mGluRs, Bockaert and Pin, 1997), may be involved in the elevation of intracellular calcium, as InsP3 triggers calcium release from internal stores via InsP3 receptors (Berridge 1993). Bashir *et al.* (1993), found that activation of mGluRs is needed for LTP induction. Moreover, activation of mGluRs can trigger LTP even when NMDA receptors are blocked (Bortolotto and Collingridge, 1993). Internal store release may also take place in an InsP3 independent manner, via calcium induced calcium release (CICR). This takes place through ryanodine receptors in a mechanism possibly regulated by the second messenger, cADP ribose. CICR may be important in regenerating a calcium signal that is initially restricted to a small part of the neuron (for a review of CICR in neurons, see Verkhratsky & Schmigol, 1996).

As stated above, the establishment of LTP requires a critical period of transcription (Nguyen *et al.* 1994). The changes in calcium following LTP-inducing stimuli elicit the rapid induction of a number of immediate early genes (IEG). Wisden *et al.* (1990) and Cole *et al.* (1989) showed a correlation between LTP and the induction of *zif268* transcription. Worley *et al.* (1993) showed that stronger stimuli could also induce *c-fos* and *c-jun*. Such stimulus-induced gene expression was dependent on activation of NMDA receptors (Worley *et al.* 1993). Glutamate stimulation of cultured neurons triggers the transcriptional induction of a number of genes, including*fos, fos B, zif268, c-jun, nur/77* and *jun B* by a NMDA receptor mediated mechanism (Bading *et al.* 1995). IEGs are genes whose transcription can be triggered in the absence of *de novo* protein synthesis and many (all those listed above) are transcription factors. These transcription factors likely contribute to secondary waves of transcription, leading to the structural and functional changes to the neuron required for the maintenance of LTP, although the exact mechanisms underlying this are unclear. Thus, the mechanisms by which calcium activates transcription in electrically excitable cells has been the subject of much recent research. Below is a brief overview of the essentials of transcriptional activation-the point at which gene expression is most often regulated.

## Regulation of gene expression

The regulation of gene expression can occur at many levels, at the point of transcription initiation, transcription elongation, RNA processing (including alternative splicing), mRNA stability, the control of translation and of protein degradation. An important and extensively studied point of control is transcription initiation. The synthesis of mRNA is catalysed by RNA polymerase (pol) II but an large number of additional proteins are needed to direct initiation to the correct place on the gene and to catalyse the whole initiation process.

A DNA sequence near the transcription start site, called the core promoter element, is the site for the formation of the pre initiation complex (PIC), a complex of RNA pol II and proteins called basal transcription factors (reviewed in Roeder 1996). RNA pol II and the basal transcription factors are

sufficient to facilitate a considerable amount of transcription *in vitro* (called basal transcription). However, *in vivo*, basal transcription levels are often extremely low, reflecting the fact that *in vivo* the DNA containing the core promoter is associated with histones and subsequently less accessible to incoming factors. For transcription to take place, other accessory factors, called activating transcription factors (hereafter known as transcription factors) are required. These factors bind to specific DNA promoter elements, located upstream of the core promoter and enhance the rate of PIC formation by contacting and recruiting the basal transcription factors, either directly, or indirectly via adapters or coactivators (for a review see Ptashne & Gann 1997).

The ability of many transcription factors to influence the rate of transcription initiation can be regulated by signalling pathways. This provides a mechanism whereby a stimulus applied to the cell that activates a signalling pathway can result in the specific activation of a subset of transcription factors. These signalling mechanisms often involve regulatory phosphorylation events at the transcription factor level that control, for example, DNA binding affinity, subcellular localisation, or its interactions with the basal transcription machinery (reviewed by Hunter & Karin 1992; Hill and Treisman 1995a). Genes whose promoters contain binding sites for these signal-inducible transcription factors are transcribed as a result of signal-activating stimuli. As stated before, *c-fos* is one of several genes whose transcription is induced by elevated levels of intracellular calcium and this is an appropriate model with which to investigate the mechanisms whereby transcription is activated by calcium.

## Calcium-responsive DNA regulatory elements

The *c-fos* promoter has two principal calcium responsive DNA regulatory elements, the cAMP response element (CRE) and the serum response element (SRE). The CRE was first identified in the promoter of the somatostatin gene as the element required to confer cAMP inducibility on the gene (Montminy *et al.* 1986; Comb *et al.* 1986). The CRE was subsequently found in a number of other genes and is a 8 bp palindromic sequence, 5'-TGACGTCA-3'. The CRE in the *c-fos* promoter is at position -60 bp relative to the transcription start site and has sequence 5'-TGACGTTT-3'. Sheng *et al.* (1988, 1990) showed that calcium influx into PC12 cells through L-type voltage gated calcium channels could activate gene expression mediated by the CRE; other cell types such as AtT20 cells and primary hippocampal neurons behave similarly (Bading *et al.* 1993; Hardingham *et al.* 1997; Johnson *et al.* 1997). The transcription factor that can mediate activation via the CRE, CRE binding protein (CREB) was isolated as a phosphoprotein that bound the CRE on the mouse somatostatin gene (Montminy & Bilezikjian 1987) and was cloned by Hoeffler *et al.* (1988). Sheng *et al.* (1990) showed that CREB also bound the *c-fos* CRE.

The SRE was identified as an element centred at -310bp required for serum induction of *c-fos* in fibroblasts (Treisman, 1985). The SRE comprises a core element 5'-CC[A/T]$_6$GG-3' that is the binding site for serum response factor, SRF (Treisman 1987; Schroter *et al.* 1987; Norman *et al.* 1988). In addition, the SRE contains a ternary complex factor (TCF) binding site, 5'-CAGGAT-3', situated immediately 5' to the core SRE, which is bound by TCF. TCF is an umbrella name for a group of Ets domain proteins, SAP-1, Elk-1 and SAP-2. These proteins cannot bind the SRE on their own but recognise the SRE/SRF complex. Like the CRE, the SRE is a target for calcium signalling pathways, and can confer calcium inducibility onto a minimal *c-fos* promoter in response to activation of L-type calcium channels (Bading *et al.* 1993; Misra *et al.* 1994; Johnson *et al.* 1997) and NMDA receptors (Bading *et al.* 1993).

## Gene regulation by spatially distinct calcium signals

Calcium is the principal mediator of electrical activity-dependent changes in neuronal gene expression. However, neurons don't respond stereotypically to electrical activation but, depending on the strength and/or duration of the stimulation, activate distinct programs of gene expression (Worley *et al.* 1993; Impey *et al.* 1996). How can a single second messenger relay a range of different types of stimulation to generate diverse patterns of transcriptional responses? The finding that stimulation of cytosolic calcium transients are often accompanied by increases in the concentration of nuclear calcium raises the possibility that spatially distinct calcium signals have different functions in the regulation of gene expression. The role of nuclear and cytoplasmic calcium signals in the control of transcription was investigated by Hardingham *et al.* (1997). The strategy used in this study was to introduce, by microinjection,

a non-diffusible calcium chelator into the nucleus of AtT20 cells. The calcium chelator was BAPTA linked to a 70 kDa dextran molecule (BAPTA-D70) which prevented diffusion of BAPTA out of the nucleus. In cells microinjected into the nucleus with BAPTA-D70, activation of calcium entry through L-type voltage-gated calcium channels still caused cytoplasmic calcium transients but increases in nuclear calcium concentrations were inhibited. Analysis of calcium-induced transcriptional responses in BAPTA-D70 microinjected cells revealed that nuclear calcium controls gene expression through the CRE, while calcium-activated transcription via the SRE is triggered in the cytoplasm and can function independently of increases in nuclear calcium concentrations (Hardingham *et al.* 1997; Bading *et al.* 1997). This study further identified CREB as a nuclear calcium-responsive transcription factor. These findings show that the intracellular localisation of calcium transients profoundly influences the transcriptional response and has particular relevance to the establishment of long term memory with which, as is outlined below, CREB-dependent transcription is inextricably linked.

## The importance of CREB in long-term memory

CREB appears to have an important role in long term memory and long lasting, protein synthesis dependent LTP. The modulation of CREB levels has been achieved through the intrahippocampal infusion of antisense oligonucleotides designed to bind and trigger degradation of CREB mRNA. Short term spatial memory was unaffected but long term memory was blocked (Guzowski and McGaugh 1997). Consistent with the model of *de novo* protein synthesis being necessary for long-term memory, inhibition of protein synthesis blocks spatial memory tested 24 hr after training, without affecting short term memory tested after 1 hr (Abel *et al.* 1997).

CREB-dependent transcription also appears to be required for the cellular events underlying long-term memory in other organisms. Studies carried out on long term facilitation (LTF) of the gill withdrawal reflex in the marine snail, *Aplysia californica* and long term memory (LTM) in the fruit fly, *Drosophila melanogaster* both represent the physiological expression of neuronal plasticity (Bailey & Kandel 1994; Tully 1994). Evidence for the involvement of CREB is strong but it is thought that cAMP and not calcium is the critical second messenger in these cases (Drain *et al.* 1991; Byrne *et al.* 1993).

The model for learning in the marine snail *Aplysia californica* is based on sensitisation of its gill withdrawal reflex, in response to a noxious stimulus (Carew *et al.* 1981). Like LTP, the process comprises two phases, a short term facilitation (STF) that lasts from minutes to a few hours after training and a long-term facilitation that lasts for hours up to a day or more (Kandel & Schwartz, 1982; Montarolo *et al.* 1996). The establishment of LTF, but not STF was found to be blocked both by inhibitors of transcription and of protein synthesis. Injection of oligonucleotides containing the CRE into the nucleus of the sensory neuron blocked LTF, without affecting STF (Dash *et al.* 1990). Thus, CREB and CRE-dependent gene expression were shown to be central to the generation of LTF.

Molecular genetic studies of behavioural plasticity in *Drosophila melanogaster* are based on studying shock-paired odour avoidance, a form of associative learning. Yin *et al.* (1995) cloned dCREB2, a fly homologue of rat CREB that is alternatively spliced to yield one isoform that acts as a cAMP responsive activator of CRE-mediated gene expression and one that acts as a repressor. Transgenic flies were subsequently produced that over-expressed either the activator or repressor isoform. Induction of the repressor isoform of dCREB2 prior to behavioural training blocked the protein synthesis dependent formation of long-term memory (LTM) induced by spaced training, without affecting early memory formation or learning. Conversely, when the activating isoform of dCREB2 was induced, it accelerated LTM formation, resulting in full induction after a single training trial which normally only activates short term memory (Yin *et al.* 1995b). Activation of CREB has also been found to be necessary for functional increases in synaptic transmission (Davis *et al.* 1996).

These studies underline the importance of elucidating the mechanisms by which calcium activates CREB-mediated transcription. As stated earlier, evidence suggests that this process is dependent on elevation of nuclear calcium, but what is the molecular mechanism of this activation process and what signalling molecules are involved?

## The mechanism of CREB activation involves a crucial phosphorylation event

CREB can bind to the CRE even prior to the activation of CRE-dependent gene expression, indicating that regulation of its activity is not via the control of its DNA binding activity (Sheng *et al.*

1990a). CREB binds the CRE as a dimer, mediated by a leucine zipper motif (Yamamoto *et al.* 1988). To activate CREB-mediated transcription, CREB must become phosphorylated on serine 133 (Gonzalez and Montminy 1989) in a process that could be triggered by elevated levels of cAMP (Gonzalez and Montminy 1989), which activates PKA.

Sheng *et al.* (1990) showed that elevation of intracellular calcium, following depolarisation of PC12 cells, resulted in CREB phosphorylation on serine 133 and activation of CREB-mediated gene expression (Sheng *et al.* 1991). CREB-mediated gene expression was abolished by mutating serine 133 to an alanine, underlining the importance of the site as a point of control by calcium signalling pathways. These results showed that CREB is a calcium responsive transcription factor (not just cAMP-responsive) and led to the assumption that CREB-mediated gene expression was triggered solely by phosphorylation of CREB on serine 133.

## Calcium-dependent signalling molecules capable of phosphorylating CREB on serine 133

### *CaM kinases and their role in calcium-activated, CRE-dependent gene expression*

CREB phosphorylation on serine 133 can be mediated by a number of protein kinases, including the multifunctional calcium/calmodulin dependent protein kinases (CaM kinases) I, II and IV (Sheng *et al.* 1991; Sun *et al.* 1994; Matthews *et al.* 1994). CaM kinases play a role in diverse biological processes such as secretion, gene expression, LTP, cell cycle regulation and translational control (for a review, see Schulman 1993). A role for CaM kinases in the calcium activation of *c-fos* expression is indicated by the attenuation of L-type calcium channel-activated *c-fos* expression in neurons by the CaM kinase inhibitor, KN-62 (Bading *et al.* 1993), and by the blocking of calcium dependent *c-fos* expression by the calmodulin antagonist, calmidazolium (Morgan & Curran 1986; Bading *et al.* 1993).

CaM kinase II, a protein highly expressed in the nervous system (Lin *et al.* 1987), was found to play an important role in LTP. Microinjection of the auto inhibitory peptide (see below) was found to block LTP (Malinow *et al.* 1989), while targeted mutation of the a isoform of CaM kinase II in mice resulted in impaired spatial learning and poor induction of LTP in brain slices of the mutant mice (Silva *et al.* 1992a, b). Expression of an activated form of CaM kinase II in the forebrain of mice resulted in the loss of hippocampal LTP and when expression was restricted to the lateral amygdala and the striatum but no other forebrain structures, there was a deficit in fear conditioning (Mayford *et al.* 1996). CaM kinase IV is similar in sequence to CaM kinase II's catalytic domain and was discovered by Ohmstede *et al.* (1989). It is expressed mainly in neuronal cells, including the cerebellum and the hippocampus (Jensen *et al.* 1991; Hardingham *et al.* 1998), in AtT20 cells (Hardingham *et al.* 1998) and has been shown to be mainly localised to the nucleus (Jensen *et al.* 1991; Bito *et al.* 1996).

Regulation and structural organisation of CaM kinases II and IV are broadly similar (for a review, see Schulman 1993; Ghosh & Greenberg 1995; Heist & Schulman 1998). Both have a N-terminal catalytic domain and a central calcium/calmodulin binding regulatory domain. The regulatory domain contains an auto-inhibitory motif that renders the active site of the enzyme inaccessible. Removal of this auto-inhibitory motif makes both CaM kinase II and IV constitutively active (Cruzalegui *et al.* 1992; Cruzalegui & Means 1993). Note that the kinase itself does not bind calcium, activation of the enzyme occurs when calcium complexed with a small protein, calmodulin, binds and displaces the auto-inhibitory domain. CaM kinase II then undergoes a number of autophosphorylation events (Miller & Kennedy 1986) and as a result the enzyme traps calmodulin and continues to be active even after calcium levels decline (Meyer *et al.* 1992). In this state, the enzyme becomes autonomous and can be said to have a short term molecular 'memory', enabling its activity to be sustained between, for example, calcium oscillations (De Konick & Schulman 1998).

Recently, autophosphorylation of a CaM kinase II at threonine 286 has shown to be important in LTP and learning (Giese *et al.* 1998). Mice with a mutation in threonine 286 of CaM kinase II showed no spatial learning and had no NMDA receptor dependent LTP in the hippocampal CA1 area. As predicted, calcium dependent activity of a CaM kinase II was unaffected by this mutation, only calcium independent activity was abolished. Thus the phenotype observed underlines the striking importance of this enzyme's calcium independent activity or 'molecular memory'.

Despite their structural similarities and their ability to phosphorylate CREB on serine 133, CaM kinases II and IV have very different effects on

CRE/CREB-mediated gene expression. Matthews *et al.* (1994) showed that a constitutively active form of CaM kinase IV, but not an active form of CaM kinase II could activate CRE-dependent transcription. Sun *et al.* (1994) further investigated the different profile of gene activation by CaM kinase II and IV. As before, it was found that CaM kinase IV was a far better activator of CREB-mediated transcription than CaM kinase II. Phosphopeptide mapping of CREB phosphorylated *in vitro* by CaM kinase II and IV revealed that CaM kinase II phosphorylates an additional site on CREB, serine 142. Phosphorylation of this site appears to have an inhibitory affect on CREB-mediated gene expression, since CaM kinase II could activate gene expression mediated by a mutant of CREB that had an alanine instead of a serine at position 142.

Thus CaM kinase IV appears to be a prime candidate for the activation of CREB-mediated gene expression by nuclear calcium signals, being located largely in the nucleus and able to efficiently activate CREB. Indeed, antisense oligonucleotide-mediated disruption of CaM kinase IV expression suppressed calcium-activated CREB phosphorylation in hippocampal neurons (Bito *et al.* 1996). Unpublished results in our laboratory suggest that this kinase is both necessary and sufficient for calcium-activated CRE/CREB-mediated gene expression. The activation of CaM kinase IV by nuclear calcium and subsequent phosphorylation of CREB may be augmented by the translocation of calmodulin to the nucleus (Deisseroth *et al.* 1998)

### *Other signalling molecules capable of phosphorylating CREB on serine 133*

CREB phosphorylation on serine 133 can be mediated by signalling molecules other than PKA and CaM kinases. Growth factors such as nerve growth factor (NGF) can activate CREB phosphorylation by a mechanism mediated by the Ras/Mitogen Activated Protein Kinase (ERK) pathway. NGF treatment of PC12 cells resulted in the Ras/MAP kinase (ERK) dependent activation of a CREB kinase (Ginty *et al.* 1994) found to be the a member of the previously identified pp90 RSK family, RSK2 (Xing *et al.* 1996). RSK2 was able to mediate CREB phosphorylation *in vivo* and *in vitro*. The fact that NGF cannot efficiently activate CRE-dependent transcription (Bonni *et al.* 1995; Johnson *et al.* 1997) demonstrates that CREB phosphorylation on serine 133 is not sufficient to activate CREB-mediated gene expression, additional activating steps are required (see below).

The Ras/MAP kinase (ERK) pathway is also activated by calcium (Bading & Greenberg 1991; Rosen *et al.* 1994) and so it is likely that RSK2 is activated by calcium signals as well as growth factors. Indeed, unpublished data in our laboratory points toward the calcium activation of both MAP kinase (ERK) and CaM kinase dependent pathways in parallel, either of which can activate CREB phosphorylation.

Another pathway has been characterised that results in CREB phosphorylation on serine 133, induced by fibroblast growth factor (FGF) and cellular stress in a neuroblastoma cell line (Tan *et al.* 1996). The CREB kinase identified was MAP kinase-activated protein kinase-2 (MAPKAP kinase-2), an enzyme that is activated by p38 MAP kinase (Rouse *et al.* 1994). p38 MAP kinase is activated by calcium signals in AtT20 cells (F Cruzalegui and H Bading, unpublished results) and also by glutamate treatment of neurons (Kawasaki *et al.* 1997) and so the p38 MAP kinase/MAPKAP kinase-2 is the third candidate pathway for mediating the calcium-activated phosphorylation of CREB on serine 133.

## Uncoupling of CREB phosphorylation from CREB-mediated transcription

CREB phosphorylation on serine 133, while necessary for CREB to function as a transcriptional activator, is not sufficient for induction of gene expression. A wide variety of extracellular signals lead to CREB phosphorylation on serine 133, but many of them, including stimulation with nerve growth factor (NGF) or epidermal growth factor (EGF), are poor activators of CRE-mediated transcription (see above). Further experiments demonstrate that serine 133 phosphorylation is not sufficient for calcium-induced CREB-mediated transcription: Nuclear calcium chelation and CaM kinase inhibition both block CRE-mediated gene expression without inhibiting CREB phosphorylation (Hardingham, Chawla, and Bading, unpublished results).

Thus, CRE-dependent transcription requires additional activation events that are provided by nuclear calcium signals and CaM kinase activity but not, for example, by NGF or EGF treatment, activators of the Ras/MAP kinase pathway. This fact is further reinforced by the observation that both activated CaM kinase IV and activated Ras trigger CREB phosphorylation but that only CaM kinase IV could activate CREB-mediated transcription (Hardingham, Chawla, and Bading, unpublished results).

We propose that this additional activating step involves the coactivator, CBP.

## The molecular mechanism by which CREB activates transcription: coactivation by CREB binding protein, CBP

As stated earlier, CREB is regulated by modification of its transactivation domain, rather than its subcellular localisation or DNA binding activity. The inducible part of the CREB transactivation domain, the kinase inducible domain (KID) assists in activating transcription initiation complex formation via its association with the coactivator, CREB-binding protein (CBP) and p300, a related protein. CBP and p300 function as coactivators for many signal dependent transcription factors such as Jun (Arias *et al.* 1994; Bannister & Kouzarides 1995), interferon-α signalling through STAT2 (Bhattacharya *et al.* 1996), Elk-1 (Janknecht & Nordheim, 1996a,b), p53 (Gu *et al.* 1997) and nuclear hormone receptors (Chakravati *et al.* 1996). The association of CBP with CREB is dependent on CREB being phosphorylated on serine 133 (Chrivia *et al.* 1993; Parker *et al.* 1996). Disruption of CBP activity by the viral oncoprotein E1A suppresses c-fos upregulation by phosphorylated CREB (Janknecht & Nordheim 1996a), emphasising the importance of CBP for CREB function.

CBP's ability to stimulate transcription may be due to its ability to recruit to the promoter components of the basal transcription machinery-it has been reported to associate with TFIIB, TATA binding protein (TBP) and RNA polymerase II complex (Kwok *et al.* 1994; Swope *et al.* 1996; Nakajima *et al.* 1997). In addition, CBP has an intrinsic histone acetyl transferase (HAT) activity (Bannister and Kouzarides 1996) as well as being able to associate with other HAT proteins, p/CAF and SRC1 (Yao *et al.* 1996; Chen *et al.* 1997). Acetylation of histones on their N-termini is known to correlate with increased transcriptional activity (Hebbes *et al.* 1988; Turner & O'Neill 1995) and a recent study has shown that the HAT domain of CBP can indeed stimulate transcription (Martinez-Balbas *et al.* 1998).

## Model for nuclear calcium-regulated transcription: regulation of CBP

The purpose of CREB phosphorylation on serine 133 appears to be to recruit the transcriptional coactivator, CREB binding protein (CBP) to the promoter (Chrivia *et al.* 1993). However, recent evidence suggests that this is insufficient to activate transcription, raising the possibility that the second regulatory event critical for transcriptional activation may involve the activation of CBP.

Very little is known about how, or if, the transactivating potential of CBP is regulated. The catalytic subunit of PKA can activate CBP (Chrivia *et al.* 1993), but the mechanism of this is unknown, since a portion of CBP that is PKA inducible retains its inducibility even when its PKA phosphorylation site is mutated (Kwok *et al.* 1994). Janknecht & Nordheim (1996a) found PKA-inducible regions of CBP at the N- and C-terminal ends of CBP and found that the N-terminus could be induced by the strong activation of the Ras/MAP kinase (ERK) pathway and could be phosphorylated *in vitro* by MAP Kinase (ERK, Janknecht & Nordheim, 1996b).

Data from our laboratory suggests that CBP is indeed critical for calcium-activated CREB-dependent transcription and, moreover, that CBP activity

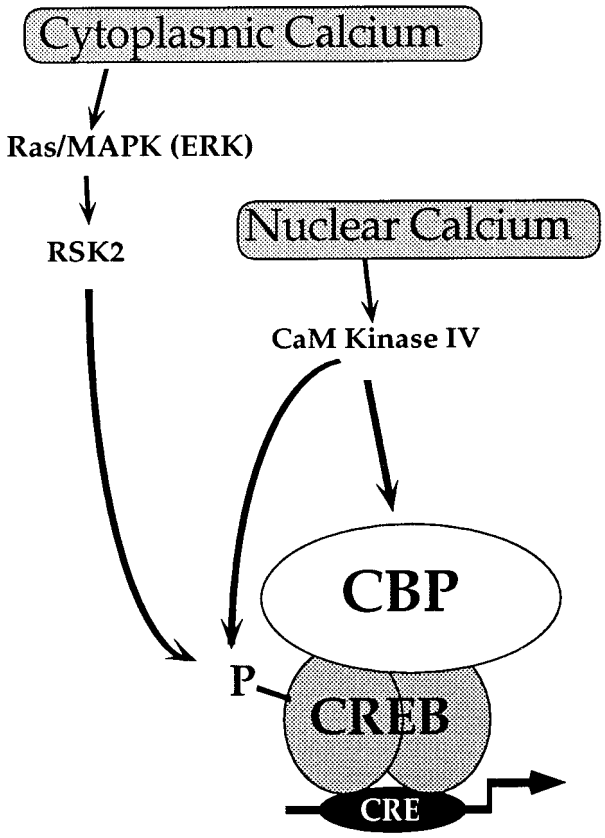


**Figure 1.** Model for calcium activation of CRE-dependent transcription.

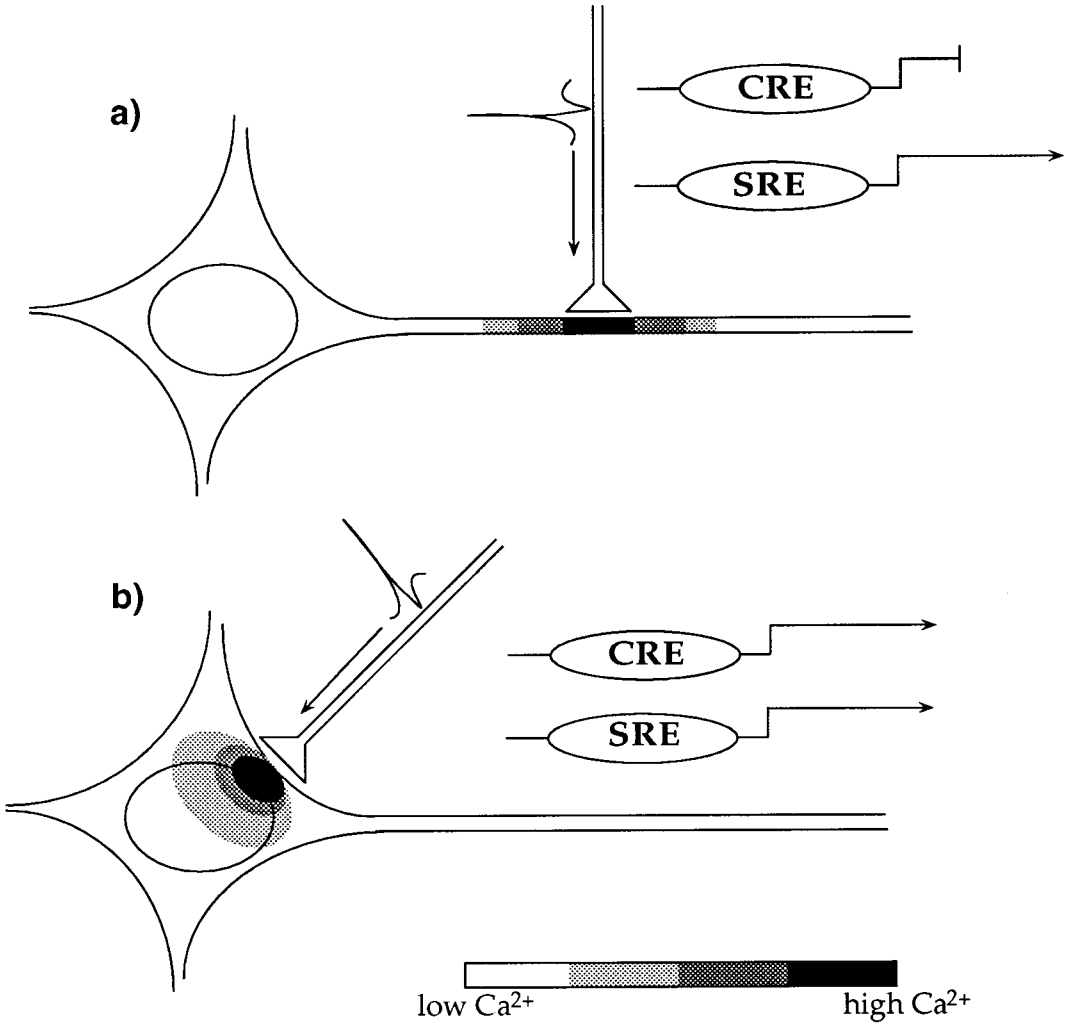


**Figure 2.** Model for the generation of spatially distinct calcium signals in neurone. a) Opening of calcium channels distal to the cell body could result in a local change in cytoplasmic calcium and activation of signalling molecules that impinge on the SRE. b) In contrast, calcium influx into the cytoplasm of the cell body likely results ih the elevation of nuclear calcium as well, activating both the SRE and the CRE.

is controlled by nuclear calcium (Chawla, Hardingham, Quinn, & Bading, unpublished results). We propose a two-step mechanism for nuclear calcium-activated CRE-dependent transcription. The first step is calcium-induced phosphorylation of CREB on serine 133; this leads to recruitment of CBP to the promoter. The second regulatory event critical for the transcriptional response stimulates the activity of CBP (Fig. 1). Both events, CREB phosphorylation on serine 133 *and* stimulation of CBP activity may be mediated by CaM kinase IV. In contrast, cytoplasmic calcium signals that activate the Ras/MAP kinase (ERK) pathway trigger CREB phosphorylation but are unable to activate CBP. This model indicates that other DNA binding proteins capable of recruiting CBP to the promoter also function as nuclear calcium-responsive transcriptional activators and may represent a general mechanism for controlling gene expression by nuclear calcium signals.

## The generation of spatially distinct calcium signals

The ability of spatially distinct calcium signals to differentially activate transcription naturally only has relevance if scenarios exist in the organism whereby nuclear and cytoplasmic calcium levels change to differing degrees. One can envisage that the length of neuronal processes allows for the generation of spatially distinct calcium signals in

response to different patterns of electrical activity in neurons. Calcium influx can take place anywhere on the neuron, calcium influx distal to the cell body may have a negligible effect on global nucleoplasmic calcium levels whereas membrane depolarisation of the cell body could result in an elevation of nuclear calcium as well as cytoplasmic calcium. Consequently, electrical input at distal dendrites may activate SRE-regulated genes, while electrical inputs near the cell body may activate SRE- and CRE-regulated genes (Fig. 2). The question of whether nuclear calcium can be regulated independently of cytoplasmic calcium is a controversial one and is discussed fully elsewhere (Santella & Carafoli 1997; Badminton *et al.* 1998). The nuclear envelope contains many of the enzymes responsible for calcium homeostasis and also those involved in phosphatidyl inositol metabolism. Certain signalling pathways, such as integrin mediated signals, cause nuclear calcium transients independent of cytoplasmic calcium (Shankar *et al.* 1993). Conversely, some cytoplasmic calcium, such as vasoconstrictor induced signals in muscle cells, appear to flow around the nucleus, leaving nuclear calcium levels relatively unchanged (Williams *et al.* 1985; Neylon *et al.* 1990).

The nucleus appears to be particularly suited to the propagation and prolongation of calcium signals. The absence of calcium buffering ATPases (take calcium up into the inter membrane space) on the inner nuclear membrane (Humbert *et al.* 1996) is thought to be behind the striking ability of elementary calcium release events proximal to the nucleus to trigger global increases in nuclear calcium concentration which last long after the cytoplasmic elementary 'trigger' has died away (Bootman *et al.* 1997; Lipp *et al.* 1997). Thus it seems plausible that spatially distinct calcium signals can exist *in vivo* although whether a semi-stable calcium gradient can exist across the nuclear envelope is another matter.

## Concluding remarks

There are a very limited number of second messengers that cells use to couple extracellular stimuli to the cell's physiological response to those stimuli. However, eukaryotic cells, and in particular neurons, are extraordinarily complex units, capable of responding to stimuli in a huge variety of subtly different ways. These two facts are in apparent conflict if one assumes that cells follow a preordained response to the elevation in intracellular concentration of a particular second messenger. The finding that spatially distinct calcium signals can differentially activate transcription provides an additional parameter that can be used by the cell to distinguish between different calcium signals.

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