REGULATION OF CALCIUM AND CYCLIC AMP SIGNALING BY THE SMALL G-PROTEIN RAP1

by

Savraj Singh Grewal

A DISSERTATION

Presented to the Cell and Developmental Biology department

and the Oregon Health Sciences University

School of Medicine

in part fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Medicine
Oregon Health Sciences University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Savraj Singh Grewal

has been approved

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Member

CONTENTS

ACKNOWLEDGMENTS

I would first like to begin by thanking all the members of the Stork lab, both past and present, for making my Ph.D. experience so enjoyable. They have always made the lab such a fun and relaxed place to work and I will miss them all very much. I would also particularly like to acknowledge Randy York, Angela Horgan, Hong Yao and Cindy Ellig for contributing to some of the work presented in this thesis. Finally, I must thank my mentor Phil Stork. Phil has always provided me with a great deal of encouragement and support throughout my graduate life. He has always allowed me to cultivate and pursue my own ideas and has fostered a very stimulating environment in which to work. Perhaps more than this, he has been a very good friend for the past four years.

I would also like to acknowledge the support and love of all my family. First and foremost, I would like to thank my parents and sister, Seema. They have shown me tremendous love and support throughout my life and I know I could never have got this far and achieved this goal without them. Although they have been far away from me during my graduate work, they have always been close to my heart.

I would also like to acknowledge my parents-in-law and Mandi and Sunny. They have always been a constant source of encouragement, love and humor throughout the last five years. In particular, my father-in-law, Harbhajan Sandhu has continually given me great encouragement and motivation for which I am truly thankful.

Finally, I would like to thank the two most important people in my life, Harpreet and Ryan. Throughout the trials and tribulations that inevitably make up the life of a graduate student, they have provided me with a constant and much needed source of joy, fun and love. I love them both so very much.

ABSTRACT

Two major intracellular signals that regulate neuronal function are calcium and cAMP. For example, both activation of G-protein coupled receptors linked to cAMP elevation and stimulation of calcium influx via either receptor- or voltage-operated calcium channels are involved in the activity-dependent regulation of neuronal events such as cell survival and synaptic plasticity. Calcium and cAMP can exert rapid actions on neurotransmission and neuronal excitability. However, in many cases, the actions of these two second messengers involve slower, long-term changes in gene expression. These effects are often mediated by stimulation of multiple intracellular signal transduction pathways. One such pathway is ERK signaling cascade. Over the last decade, it has become well established that ERKs are important in the regulation of processes such as cell proliferation and differentiation. These effects have been best studied in the context of growth factor signaling. However, an emerging theme is that ERK signaling is important in controlling various processes in mature neurons such as gene expression, neuronal excitability and synaptic plasticity. As such a great deal of attention has focused on the regulatory mechanisms that control ERK signaling in neurons. Work in our laboratory has previously described a novel ERK pathway. This involves the PKA-dependent activation of the Ras-related small G-protein, Rap1, and subsequent stimulation of the neuronal Raf isoform, B-Raf. Previoius work in our laboratory has shown that this pathway is involved in cAMP signaling. The experiments presented in this thesis suggest that this Rap1:B-Raf pathway may be important in controlling the actions of neuronal calcium as well. Using the PC12 neuronal cell model, we have demonstrated that calcium influx through depolarization-mediated opening of L-type voltage operated channels can stimulate Rap1:B-Raf signaling pathway. Like cAMP, activation of this pathway occurs via PKA. Moreover, in PC12

cells, and perhaps hippocampal neurons, activation of this pathway by calcium leads to the stimulation of ERKs. Thus, signaling through Rap1 is a common mechanism by which both calcium influx and cAMP can signal to ERKs in PC12 cells. We further examined the contribution of the Rap1-ERK pathway to the control of gene transcription by calcium influx and cAMP. One well studied transcriptional target of both calcium and cAMP signaling is the transcription factor CREB. Using the PC12 cell model system, we found that calcium influx and cAMP both stimulated CREB-dependent transcription via a Rap1-ERK pathway, but this regulation occurred through distinct mechanisms. Calcium-mediated phosphorylation of CREB through the PKA-Rap1-ERK pathway. In contrast, cAMP phosphorylated CREB via PKA directly but required a Rap1-ERK pathway to activate a component downstream of CREB phosphorylation and CBP recruitment. These data suggest that the Rap1/B-Raf signaling pathway may have an important role in the regulation of CREB-dependent gene expression in neurons. Taken together, the results from this thesis indicate that Rap1 represents an important target for both calcium and cAMP signaling in neurons.

CHAPTER ONE

INTRODUCTION

Neuronal Actions of Calcium and Cyclic AMP

Calcium Signaling in Neurons

Increases in intracellular calcium levels can elicit wide array of effects in both excitable and non-excitable cells (Clapham, 1995). As such, there are numerous control mechanisms to ensure the precise regulation of intracellular calcium levels. In neurons, a major function of calcium elevation is to link changes in electrical excitability to the stimulation of specific intracellular signaling enzymes and pathways (Berridge, 1998)(Fig 1.1). The best-described mechanism to elevate neuronal calcium levels involves calcium influx through various plasma membrane ion channels. For example, membrane depolarization can induce calcium influx through a family of voltage operated channels (P, N, T and L-type channels) (Artalejo, 1997). These calcium fluxes are important in regulating action potentials, neurotransmitter release and post-synaptic signaling responses (Berridge, 1998). Neurotransmitters are also able to stimulate calcium influx. For example the NMDA class of receptor for the excitatory neurotransmitter, glutamate, is a calcium channel. Pre-synaptic release of glutamate is able to bind to post-synaptic NMDA (N-methyl -D-Aspartate) receptors leading to channel opening and calcium influx. This type of synaptic communication is particularly important in mediating rapid post-synaptic signaling events and underlies many persistent neuronal adaptive responses such as long-term potentiation (LTP) and long-term depression (LTD) (Cain, 1997; Zucker, 1999).

Release of calcium from intracellular stores provides an additional mechanism by which intracellular signaling events can be initiated (Berridge, 1998). Ryanodine receptors (RYRs) and inositol 1,4,5-tris-phosphate receptors (IP3Rs) are specialized channels distributed throughout the endoplasmic reticulum (ER) and are primarily responsible for mediating this increase (Koizumi, et al., 1999). Activation of multiple classes of

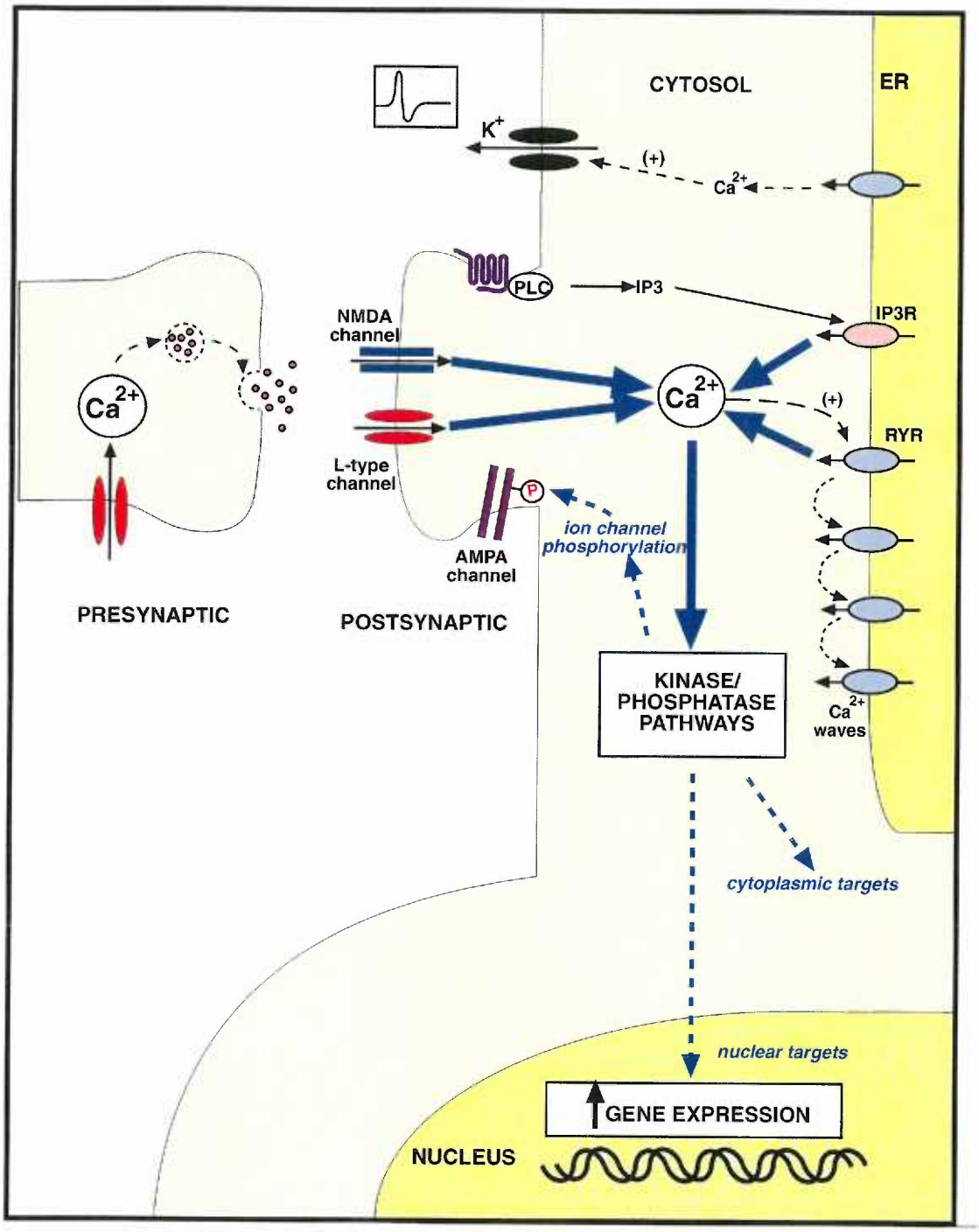

Figure 1.1. **Calcium signaling in neurons.** Elevations in calcium levels can occur through multiple mechanisms. These include both calcium influx through L-type and NMDA channels and also release of intracellular calcium stores from the ER through both ryanodine receptors (RYR) and IP3 receptors (IP3R). Increases in intracellular calcium can exert rapid effects on neuronal excitability. For example, calcium can induce presynaptic neurotransmitter release and can regulate the functions of potassium channels that control action potential firing. However, many of the actions of calcium involve the stimulation of multiple kinase/phosphatase pathways. Activation of these pathways often culminates in the regulation of gene expression (see text for details).

neurotransmitter and neuropeptide membrane receptors can elicit increases in IP3 (inositol 1,4,5-tris-phosphate) levels leading to the opening of InsP3Rs (Figure 1.1). In addition, through the process of calcium-induced calcium release, entry of calcium through either receptor- or voltage-gated ion channels can mobilize these intracellular stores (Berridge, 1998). Calcium released from these stores is important in the regulation of neuronal excitability. For example, calcium from the ER can regulate potassium channels responsible for controlling the duration and firing pattern of action potentials (Sah and McLachlan, 1991). Furthermore, these intracellular stores are the source of the well described calcium 'spikes' and 'waves' that are important for propagating calcium signals along dendrites and axons (Berridge, 1998). However, compared to our understanding of calcium influx, surprising less is known about the signal transduction pathways that can be activated by intracellular calcium stores. Nevertheless, given that the ER extends as a network throughout a cell, the release of calcium from intracellular stores may serve as a mechanism to ensure appropriate localized calcium signaling. Ultimately, the coordinated regulation of both influx and intracellular release is likely to be important in the regulation of the amplitude, duration and localization of intracellular calcium levels – factors that all influence the physiological outcome of calcium signaling (Berridge, 1998).

Elevations in intracellular calcium levels can induce rapid changes in neuronal function in the time-scale of milliseconds to seconds. Calcium can regulate neuronal excitability through the control of ion channel function (Sah and McLachlan, 1991). These actions often involve direct binding of calcium ions to the channels themselves. In addition, calcium has a well-described role in initiating the process of neurotransmitter release (Matthews, 1996). Action potential-mediated depolarization at nerve terminals is sufficient to induce calcium influx through voltage-gated ion channels. This transient rise in intracellular calcium levels can then trigger a rapid series of events leading to synaptic vesicle fusion to the presynaptic membrane and subsequent release of neurotransmitter into

the synaptic cleft (Matthews, 1996). However, in addition to these rapid actions, neuronal calcium can also induce more long-term persistent changes in neuronal function on the time-sale of minutes to hours to days. These effects involve the ability of transient increases in intracellular calcium concentrations to trigger the activation of multiple and diverse signal transduction pathways often culminating in the regulation of gene transcription (Ghosh and Greenberg, 1995). For example, following stimulation of post-synaptic NMDA receptors at excitatory synapses within the hippocampus, calcium influx can activate multiple kinase signaling pathways (Ghosh and Greenberg, 1995). These include the calcium/calmodulin-dependent kinases (CaM kinases), the extracellular-signal-regulate kinases (ERKs) and the cAMP-dependent protein kinase (PKA) (Ghosh and Greenberg, 1995). Stimulation of these various signaling cascades results in the phosphorylation of an array of cytoplasmic proteins, including ion channels, and also the initiation of gene transcription (Ghosh and Greenberg, 1995). Depending on the pattern of synaptic stimulation, the net effect of these calcium–dependent signaling events may involve a long-term change in synaptic strength (Malenka, 1994; Zucker, 1999). Elevations in neuronal calcium levels can also promote the long-term survival of multiple neuronal populations grown in culture (Franklin and Johnson, 1992). For example, depolarization–mediated calcium influx through L-type calcium channels can exert a survival effect in sympathetic neurons through activation of the Akt kinase pathway (Crowder and Freeman, 1999; Vaillant, et al., 1999). Similarly, calcium influx can prevent cell death in cerebellar granule neurons by upregulating gene expression via the transcription factor MEF2 (Mao, et al., 1999).

The nature of these calcium-mediated signal transduction pathways differs depending on factors such as cell-type and the source, amplitude and duration of the calcium signal. However, it is interesting to note that calcium can activate an array of kinase signaling pathways (Ghosh, et al., 1994; Ghosh and Greenberg, 1995). In particular, calcium can

use a number of pathways best charcaterized as targets for growth factor signaling. For example, calcium can stimulate multiple tyrosine (e.g. Src-family, PYK2) and serine/threonine (e.g. PKA , CaM kinase, ERK, Akt) kinase cascades (Ghosh and Greenberg, 1995; Lev, et al., 1995; Rusanescu, et al., 1995). In addition, calcium can signal through lipid kinases such as phoshoinositide-3-kinase (PI3K) (Vaillant, et al., 1999). Calcium can also activate various intracellular phosphatases including the serine/threonine phosphatase, calcineurin (Bito, et al., 1996; Graef, et al., 1999). The mechanisms by which calcium can signal through these various signal tranduction cascades are complex and are only beginning to be understood. However, numerous studies have demonstrated that these pathways control many of the short- and long-term actions of neuronal calcium (Bito, et al., 1997; Ghosh and Greenberg, 1995). Given the extensive and well–described cross talk between many of these signaling pathways, it is apparent that calcium is a versatile second messenger capable of initiating a diverse array of signaling events. In addition, since other signaling molecules such as growth factors and hormones use many of these pathways, these cascades may allow for potential cross talk and synergy between calcium and growth factor/hormone signaling (Vaillant, et al., 1999). Understanding aspects of this potential cross talk forms the basis of part of this thesis (see chapter 3).

Cyclic-AMP signaling in neurons

Like calcium, cAMP is a ubiquitous second-messenger capable of controlling multiple events in both excitable and non-excitable cells (Blitzer, et al., 1995; Della Fazia, et al., 1997; Dumont, et al., 1989; Epstein, et al., 1975; Frey, et al., 1993; Krebs and Beavo, 1979; Rydel and Greene, 1988; Whitfield, et al., 1979). Elevations in intracellular cAMP levels are achieved through the stimulation of the multi-gene family of adenylate cyclases proteins (Houslay and Milligan, 1997). The classically described mechanism by which

adenylate cyclases are activated involves stimulation of hormone or neurotransmitter receptors coupled to the Gs family of heterotrimeric receptors (Simonds, 1999). Upon receptor activation, the Gs alpha subunit is released and it directly binds to an intracellular domain present in all adenylate cyclases. This binding stimulates cyclase activity leading to the generation of cAMP (Simonds, 1999). Certain classes of adenylate cyclase are also regulated by calcium signaling as discussed below (Xia and Storm, 1997). Another class of enzyme, the cAMP phosphodiesterases, is responsible for the degradation of cAMP (Soderling and Beavo, 2000). There are multiple members of both the adenylate cyclases and phosphodiesterase families of proteins, many having different cell-type expression patterns and distinct subcellular localization. The regulation of these two classes of enzymes is therefore a very powerful way to fine-tune cAMP responses within a cell.

The major downstream target for cAMP signaling is the cAMP-dependent protein kinase or protein kinase A (PKA) (Houslay and Milligan, 1997). The PKA holoenzyme consists of a dimer of two cAMP–binding regulatory (R) subunits bound to two catalytic (C) subunits (Figure 1.2). There are three known isoforms of the C subunit (α, β and γ) each with similar enzymatic activities and localization (Houslay and Milligan, 1997). In contrast, the major R subunit isoforms (RIα and β, and RIIα and β) exhibit very different localization patterns within the cell. The type I holoenzyme (RI, C) is almost exclusively cytosolic, while the type II holoenzyme (RII, C) is associated with membranes, organelles and particulate cell fractions (Houslay and Milligan, 1997). The subcellular targeting of the type II holoenzyme may be achieved in large part by the binding of RII to A–kinase anchoring proteins (AKAPs) (Colledge and Scott, 1999). This family of functionally related proteins binds specifically to RII subunits and, by virtue of distinct targeting domains within their structure, localize the holoenzyme to discrete regions within a cell (e.g. plasma membrane, perinuclear regions, vesicles, post-synaptic sites etc.). The

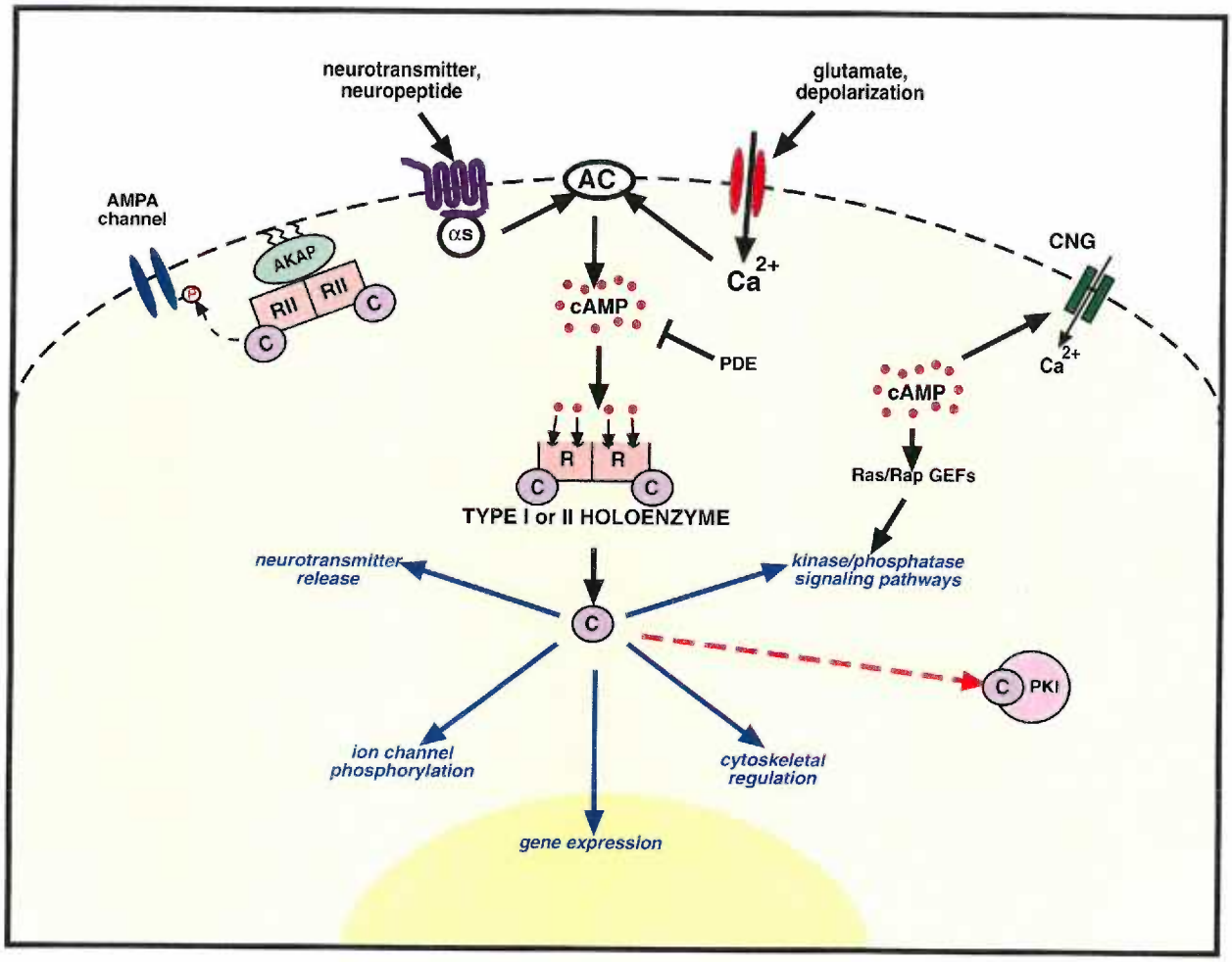

Figure 1.2. **Cyclic AMP signaling in neurons.** Elevations in cAMP levels can occur through both G-protein coupled receptor activation and calcium influx. Both stimuli can activate adenylate cyclases (AC). The best described target for cAMP is PKA. Once activated PKA can regulate a number of neuronal functions, including gene expression. These effects are often controlleD by PKA targeting via the AKAP family of anchoring proteins. The actions of PKA are also regulated by an inhibitory protein, PKI, that can bind to and sequester free catalytic (C) subunit. Other targets of cAMP include Ras and Rap GEFs and also the cyclic-nucleotide gated calcium channels (CNG). AC, adenylate cyclase; PDE, phosphodiesterases.

targeting of the PKA holoenzyme in this manner may be important for the generation of PKA signal specificity. For example, two AKAPS, yotaio and AKAP 79 are involved in targeting PKA to glutamate receptors (Colledge, et al., 2000; Fraser and Scott, 1999; Rosenmund, et al., 1994; Westphal, et al., 1999). Thus upon cAMP elevation, PKA can efficiently phosphorylate these receptors and regulate their channel properties. Given that any one cell is likely to exhibit an array of AKAP-PKA interactions, targeting of PKA to distinct substrates may be one mechanism to ensure appropriate and specific phosphorylation in response to cAMP stimulation.

The co-operative binding of two cAMP molecules to each of the R subunit dimers stimulates the enzymatic activity of the PKA holoenzyme (Taylor, et al., 1990). This decreases their affinity for the C subunits, allowing them to dissociate and diffuse away. The free C subunits are then capable of phosphorylation of specific substrate serine /threonine residues (Taylor, et al., 1990). Within neurons, PKA activity can mediate an array of both rapid and sustained effects. As previously discussed, PKA can directly phosphorylate a number of different ion channels, thus regulating their channel properties (Fraser and Scott, 1999). These actions, in part, account for the ability of neurotransmitter-mediated elevations in cAMP to rapidly affect neuronal excitability. PKA can also stimulate neurotransmitter release, possibly by a direct phosphorylation of components of the secretory machinery (Matthews, 1996). Elevations in cAMP levels can also induce longer-term, persistent changes in neuronal function. Examples of these long-term changes include the regulation of synaptic plasticity through processes such as LTP (Abel, et al., 1997), the maintenance of cell survival (Rydel and Greene, 1988), the stimulation of axonal and dendritic outgrowth (Lohof, et al., 1992; Ming, et al., 1997; Song, et al., 1997; Zheng, et al., 1994) and the regulation of neuronal phenotype (Galter and Unsicker, 2000). As with calcium signaling, the majority of these actions involve the ability of cAMP, through PKA, to stimulate multiple intracellular signal transduction

pathways leading to the stimulation of gene expression. For example, PKA itself is able to stimulate the activity of a number of transcription factors such as CREB(Montminy, et al., 1990) and also regulate other components of the cell transcriptional machinery , for example histone H3 (Daniel, et al., 1998; DeManno, et al., 1999). These actions occur largely through direct PKA-dependent phosphorylation and provide a direct mechanism by which cAMP can influence the transcriptional activity of a cell. In addition, it is clear that cAMP and PKA are able to modulate cell function through cross talk with other signaling pathways. A central tenet of this thesis is that the ability of PKA to cross-talk with the ERK signaling pathway is important in controlling the regulation of gene expression by cAMP (see chapter 4).

Although virtually all cAMP-mediated responses within a cell occur through PKA, there are two notable examples of non-PKA actions of cAMP within neurons. The first involves a class of cyclic-nucleotide gated ion channels (CNGs) (Zagotta and Siegelbaum, 1996). These are particularly important in controlling sensory neuron function where they represent the final transduction steps in the processes of olfaction and vision. For example, CNGs present in the cilia of olfactory neurons re primarily responsible for the odorant-induced increase in calcium ion flux that triggers neuronal activation (Zagotta and Siegelbaum, 1996). A second example of a non-PKA action of cAMP involves a class of guanine nucleotide exchange factor (epacs or cAMP-GEFs) (de Rooij, et al., 1998; Kawasaki, et al., 1998). These, proteins are highly expressed in neurons and stimulate the GTP-loading of Rap1, a small G-protein, in response to direct cAMP binding (see chapter 2).

Cross-talk between cAMP and calcium signaling

Elevations in intracellular calcium and stimulation of cAMP levels through activation of Gs-coupled neurotransmitter receptors can often elicit similar effects in neurons. For example, both cAMP and calcium can cooperate to regulate processes such as neuronal cell survival and synaptic plasticity (Blitzer, et al., 1995; Goldberg and Barres, 2000). These actions may reflect signaling through intracellular pathways converging on a common target. An example is the ability of both neurotransmitter receptors and calcium influx to stimulate a class of adenylate cyclase and elevate cAMP levels (Impey, et al., 1994). These adenylate cyclases (types I and VIII) can be stimulated by either binding of the Gαs subunit following receptor activation or by binding to calmodulin following increases in intracellular calcium levels (Houslay and Milligan, 1997). Type I and VIII adenylate cyclase are highly expressed within discrete regions of the CNS and can therefore act to integrate both Gs-coupled receptor and calcium signaling in neurons (Xia, et al., 1991). Moreover, this integration of two signaling pathways often occurs in a synergistic manner with calcium and receptor activation together leading to robust activation of cAMP signaling (Impey, et al., 1994). The importance of these adenylate cyclases has been demonstrated in mouse 'knockout' studies in which deletion of these enzymes disrupts components of synaptic plasticity. For example, Storm and colleagues have recently demonstrated that mice lacking both the type I and type VIII adenylate cyclases exhibit defects in hippocampal LTP and have impaired performance in behavioural models of learning (Wong, et al., 1999). Signaling through these enzymes may therefore provide a mechanism by which calcium and cAMP signaling can converge onto a common target, PKA. Moreover, this further suggests that calcium and cAMP can activate similar PKA-dependent pathways. One hypothesis of this work is that calcium influx and cAMP stimulation can both activate a

common PKA-dependent pathway leading to the activation of ERKs and stimulation of gene expression in neurons (see chapters three and four).

Regulation of neuronal gene expression by calcium and cAMP

Calcium and cAMP can regulate many intracellular signaling pathways as described above. One target for these pathways is the regulation of gene expression. Accumulating evidence suggests that calcium and cAMP-mediated signaling pathways can regulate the activity of a wide range of transcription factors (Daniel, et al., 1998; Ghosh and Greenberg, 1995). Indeed, changes in transcription factor activity underlie many of the physiological actions of cAMP and calcium in both neuronal and non-neuronal cells. For example, in lymphocytes, elevations in intracellular calcium can regulate the activity of NFAT (Nuclear Factor of Activated T-cells), a transcription factor important in controlling the T-cell responsiveness to antigen presentation (Crabtree, 1999). Moreover, in liver cells, glucagon-mediated increases in cAMP levels are responsible for the stimulation of PEPCK gene expression, an enzyme important in glucose metabolism. This increase in gene expression involves the cAMP-induced regulation of transcription factors such as C/EBP (CCAAT Enhancer Binding Protein) and CREB (cAMP-Responsive Element Binding protein) (Roesler, 2000; Roesler, et al., 1994).

The ability of both calcium and cAMP to increase gene expression is thought to underlie their induction of long-term changes in neuronal function. For example, activity-dependent increases in synaptic strength are, in part, associated with cAMP- and calcium-mediated increases in the expression of various ion channels and cell adhesion molecules (Martin and Kandel, 1996; Nayak, et al., 1998). Furthermore, these transcription-dependent changes in synapse function are thought to form the molecular bases for long-term memory (Bailey, et al., 1996). A great deal of attention has therefore focused on examining the mechanisms

by which both calcium and cAMP can regulate transcription in neurons. Current evidence indicates that both stimuli can regulate the activity of many different nuclear transcription factors via the stimulation of a diverse array of cytoplasmic signal transduction pathways (Daniel, et al., 1998; Ghosh, et al., 1994; Ghosh and Greenberg, 1995; Montminy, et al., 1990). In the majority of cases, the activity of these transcription factors is regulated by calcium- and cAMP-mediated phosphorylation . For example, the Ets family of transcription factors can be phosphorylated and activated by ERKs . Given the ability of both calcium and cAMP to activate ERKs (see below), these transcription factors have been demonstrated to be targets of activity-dependent neuronal signaling (Xia, et al., 1996). The serum-response factor (SRF) and the transcription factors of the AP1 complex (Jun and Fos) are also targeted for phosphorylation by calcium and cAMP signaling pathways (Cruzalegui, et al., 1999; Miranti, et al., 1995; Misra, et al., 1994). The activation of all these transcription factors has been well characterized within the PC12 cell line model. Moreover, stimulation of these trancription factors by variety of different neurotransmitters that can elevate either calcium or cAMP levels has been well established in many neuronal systems (Sgambato, et al., 1998; Sgambato, et al., 1998; Vanhoutte, et al., 1999). However, recent studies have shown that both cAMP and calcium can also regulate transcription factors that have been best characterized in non-neuronal cells. For example, calcium influx in neurons can regulate gene expression through NFAT, a transcription factor best characterized in lymphocytes (Graef, et al., 1999). Moreover, both cAMP and calcium have been shown to control the transcriptional activity of C/EBP in hippocampal cells (Taubenfeld, et al., 2001; Yukawa, et al., 1998) and *Aplysia* sensory neurons (Alberini, et al., 1994). In both cases, regulation of C/EBP appears to be important for the induction of synaptic plasticity.

An important challenge now is to begin to understand how the coordinated actions of these various transcription factors can increase gene expression in response to cAMP and

calcium. Important insights have come from the study of immediate-early gene induction by both stimuli (Curran and Morgan, 1995; Morgan and Curran, 1989; Sheng, et al., 1993). For example, stimulation of the Fos gene by both cAMP and calcium requires the activation of a number of transcription factors (e.g. SRF, Elk-1, CREB) bound to distinct, well-defined regions within the Fos promoter (e.g. the serum response element, SRE; the cAMP-response element, CRE) (Sassone-Corsi, et al., 1988; Sheng, et al., 1988). The increased expression of hormones, growth factors and neuropeptides (e.g. NGF and somatostatin) similarly requires the coordinated actions of multiple transcription factors bound to distinct promoter regions of these genes (Colangelo, et al., 1998; Montminy, 1997; Peers, et al., 1991; Peers, et al., 1990). In some circumstances, different transcriptional responses may be generated depending on the source of the calcium or cAMP signal. For example, in hippocampal cells, L-type channel-mediated calcium influx is much more efficient at inducing CREB-dependent transcription than NMDA receptor activation (Bading, et al., 1993). Similarly, in cortical neurons calcium influx through L-type channels is more effective at stimulating BDNF expression (Shieh, et al., 1998). These different responses of calcium influx may reflect the actions of spatially distinct pools of calcium. For example, in ATt20 cells, activation of CRE-dependent transcription requires nuclear calcium increases, while activation of the SRE only requires elevation of cytosolic calcium concentrations (Hardingham, et al., 1997). The duration and magnitude of calcium and cAMP signaling also determines the transcriptional response. Calcium and cAMP signals can additionally regulate the activity of many other proteins involved in gene expression such as transcriptional co-activators (Hardingham, et al., 1999; Kwok, et al., 1994) (see below) and components of the nucleosomal and basal transcriptional machinery (e.g. histones, TFII-I) . Hence, we are only beginning to reveal the complex series of events that control the stimulation of neuronal gene expression in response to these two simple second messengers.

CREB: a target for calcium and cAMP signaling in neurons

Perhaps the best characterized trancription factor that can be activated by cAMP and calcium signals in neurons is CREB (cAMP-responsive element binding protein) (Shaywitz and Greenberg, 1999). CREB was first identified over a decade ago (Montminy and Bilezikjian, 1987). At the time it was known that cAMP stimulation in neurons and PC12 cells could lead to increased neuropepetide gene expression (e.g. somatostatin) (Montminy, et al., 1996). Numerous studies subsequently focused on identifying regions within the promoters of these genes that could confer cAMP responsiveness. These revealed an eight base pair DNA element, designated the cAMP-responsive element (CRE), that was critical for cAMP stimulation of a reporter gene fused to the somatostatin promoter (Montminy, et al., 1986). Interestingly this CRE was also present in the upstream promoter regions of other cAMP-responsive neuropeptide genes such as proenkephalin and corticotrophin-releasing hormone (Goodman, 1990). Subsequent studies, using DNA-affinity chromatography, lead to the isolation of a protein from PC12 cells, CREB, which could bind to the somatostatin CRE (Montminy and Bilezikjian, 1987). CREB was shown to bind constitutively to the CRE as a dimer and to be stimulated by PKA-dependent phosphorylation on a single residue, serine-133, following cAMP treatment (Gonzalez and Montminy, 1989; Yamamoto, et al., 1988). Further studies identified another two transcription factors, ATF-1 and CREM, that are similar to CREB and that are now part of the CREB family (Sassone-Corsi, et al., 1988). These are structurally and functionally similar to CREB, although interestingly, one truncated isoform of the CREM family, ICER, can act as an inhibitor of CRE-mediated transcription (Sassone-Corsi, et al., 1988).

At the about the same time as these studies on cAMP signaling were being carried out, a number of laboratories were attempting to elucidate the mechanisms by which calcium

could stimulate gene expression in neuronal systems. This work stemmed from earlier findings that calcium influx in PC12 cells could rapidly stimulate the expression of the Fos proto-oncogene (Greenberg, et al., 1986; Morgan and Curran, 1986). These results were the first demonstration that calcium could induce a rapid genetic response in cells. Moreover, they began to provide a biochemical validation of an emerging view that changes in gene expression mediated by activity-dependent signaling were critical for long-term information storage in neurons (Goelet, et al., 1986). Consequently, a great deal of effort was made in determining the mechanisms by which calcium could stimulate Fos expression. A series of studies identified a calcium-responsive element (CaRE) within the Fos promoter that was very similar to the recently characterized CRE (Sheng, et al., 1988; Sheng, et al., 1990). Subsequently it was demonstrated that CREB could also bind to this CaRE and mediate the ability of calcium, and cAMP, to stimulate transcription (Sheng, et al., 1990). Like cAMP, the ability of calcium to stimulate transcription through CREB required phosphorylation of serine 133 (Sheng, et al., 1990). However, it has proved very difficult to identify the CREB kinase(s) that mediate the actions of calcium. Many different kinases have been shown to be able to phosphorylate CREB including, PKA, members of the CaM kinase family, members of the protein kinase C (PKC) family, the MAPK-activate protein (MAPKAP) family, Akt and two related kinase families, ribosomal S-kinase (RSK) and mitogen-and stress-activated kinase (MSK), that are stimulated by ERKs (Shaywitz and Greenberg, 1999). Current evidence indicates that calcium signaling can use many of these kinases to phosphorylate serine 133 of CREB. The exact kinase that is activated appears to depend on both the cell-type and the extracellular stimulus (Shaywitz and Greenberg, 1999).

Understanding the mechanism(s) by which serine-133 phosphorylated CREB can stimulate transcription has received a great deal of attention over the last decade. These studies have been spurred by the identification of CBP (CREB-binding protein) (Arias, et al., 1994;

Chrivia, et al., 1993; Kwok, et al., 1994). CBP is a 265kD molecule that can associate with the serine-133 phosphorylated form of CREB. This interaction involves the binding of a region of CREB, the kinase inducible domain (KID) containing the phosphorylated serine-133 residue, to a KID interaction domain (KIX) on CBP (Chrivia, et al., 1993; Parker, et al., 1996). Recent crystallographic studies have begun to reveal how serine-133 phosphorylation induces the association between the KID and KIX domains (Parker, et al., 1996). CREB:CBP binding appears to be critical for mediating CRE-dependent transcription. Disruption of this interaction by nuclear injection of either anti-CBP antibodies or KIX peptides abrogates transcription (Chrivia, et al., 1993). In contrast overexpression of CBP can augment PKA induced CREB transcriptional activity (Kwok, et al., 1994). However, the mechanism by which CBP promotes transcription is still unclear. One model suggests that CBP may act as a transcriptional adapter molecule. Studies have demonstrated that when bound to CREB, CBP can facilitate interactions with RNA polymersase II, by binding to proteins such as RNA helicase I (Nakajima, et al., 1997). CBP also has intrinsic histone acetyl–transferase (HAT) activity and can recruit other HAT molecules such as P/CAF (Bannister and Kouzarides, 1996; Ogryzko, et al., 1996). Given the well described effects of histone acetylation on the facilitation of gene transcription, this may provide an additional explanation for how CBP may function. It is also unclear whether the activity of CBP can be regulated by extracellular signals. However, CBP is a phosphoprotein and a number of studies have identified kinases that can both phosphorylate CBP and stimulate CBP–dependent transcription of artificial reporter genes. In particular, these actions have been described in the context of both calcium- and cAMP-mediated signaling (see chapter 4 for discussion). The regulation of CBP function may therefore provide an additional target for the regulation of transcription by these two stimuli.

CREB can be phosphorylated on other residues in addition to serine-133. For example, following calcium stimulation, CaM kinase II can phosphorylate serine-142 (Sun, et al., 1994). This phosphorylation inhibits CREB dependent transcription, possibly by disrupting CREB/CBP interaction (Parker, et al., 1998). Serine 129 can also be phosphorylated by GSK-3, but the *in vivo* consequence of this phosphorylation remains unclear (Fiol, et al., 1994). In addition to the KID domain, other regions of CREB are also critical for mediating its actions. For example, a glutamine rich domain, the Q2 domain, has been shown to interact with components of the basal transcriptional machinery such as TAF130 and TFIIB(Kee, et al., 1996; Xing, et al., 1995).

Over the last decade, the ability of both cAMP and calcium to activate CREB has been demonstrated in a number of neuronal systems (Shaywitz and Greenberg, 1999). Such studies have been aided by the availability of phosphorylation-specific antibodies, which can recognize the serine-133 phosphorylated form of CREB (Ginty, et al., 1993). These have allowed the identification of neuronal stimuli that can trigger CREB phosphorylation. For example increases in CREB phosphorylation has been observed both in dispersed and organotypic slice cultures of various neuronal populations following a variety of different stimuli such as membrane depolarization (Bito, et al., 1996; Impey, et al., 1998; Sheng, et al., 1990), NMDA receptor activation (Vanhoutte, et al., 1999) and stimulation of G-protein coupled neurotransmitter receptors (Zanassi, et al., 2001). Moreover, using immunocytochemistry of brain slices these antibodies have allowed the identification of stimuli such as seizures(Moore, et al., 1996), operant conditioning (Impey, et al., 1998; Swank, 2000), odorants (Moon, et al., 1999), and circadian rhythms (Obrietan, et al., 1999), which can induce CREB phosphorylation in distinct brain regions within whole animals. However, it is important to emphasize that CREB phosphorylation may not provide an accurate indication of transcriptional activation. For example, a number of

stimuli such, as neuroptrophins, can markedly increase CREB phosphorylation yet are very weak stimulators of CRE-dependent transcription (Shaywitz and Greenberg, 1999). Clearly, signaling events in addition to CREB phosphorylation are required to achieve full transcription (Figure 1.3). In the case of cAMP, and in particular, calcium signaling, the elucidation of these intracellular signaling pathways that are required for both CREB phosphorylation and stimulation of CRE-dependent transcription remain important areas for future research. Understanding this regulation forms the basis for part of this thesis. In particular we have examined whether the ability of both cAMP and calcium to stimulate ERKs via a PKA-dependent pathway contributes to the regulation of CREB-dependent transcription (see chapter four). This ERK pathway is described in more detail below.

Extracellular-signal-regulated kinase (ERK) signaling

The ERKs are a class of serine/threonine kinase that exhibit a wide range of effects in virtually all eukaryotic cells. They belong to the larger class of mitogen-activated protein kinases (MAP kinases), and they have been extensively studied in a number of experimental systems including, *Drosophila*, *C elegans*, yeast and mammals (Schaeffer and Weber, 1999). They have been shown to regulate events such as cell proliferation, differentiation and cell-fate determination, and apoptosis (Brunner, et al., 1994; Herskowitz, 1995; Schaeffer and Weber, 1999; Selfors and Stern, 1994). As suggested by their name, ERKs can be activated by many distinct and diverse extracellular stimuli including growth factors, hormones, steroids, neurotransmitters, cell-adhesion molecules and stress stimuli (Schaeffer and Weber, 1999). The stimulation of ERK signaling by virtually all these stimuli involves the activation of a core intracellular signaling module consisting of three enzymes, a MAP kinase kinase kinase, RAF, a MAP kinase kinase, MEK and the MAP kinase, ERK (Schaeffer and Weber, 1999). The first of these enzymes, the Raf family of serine/threonine kinase (A-Raf, B-Raf, C-Raf1) are activated

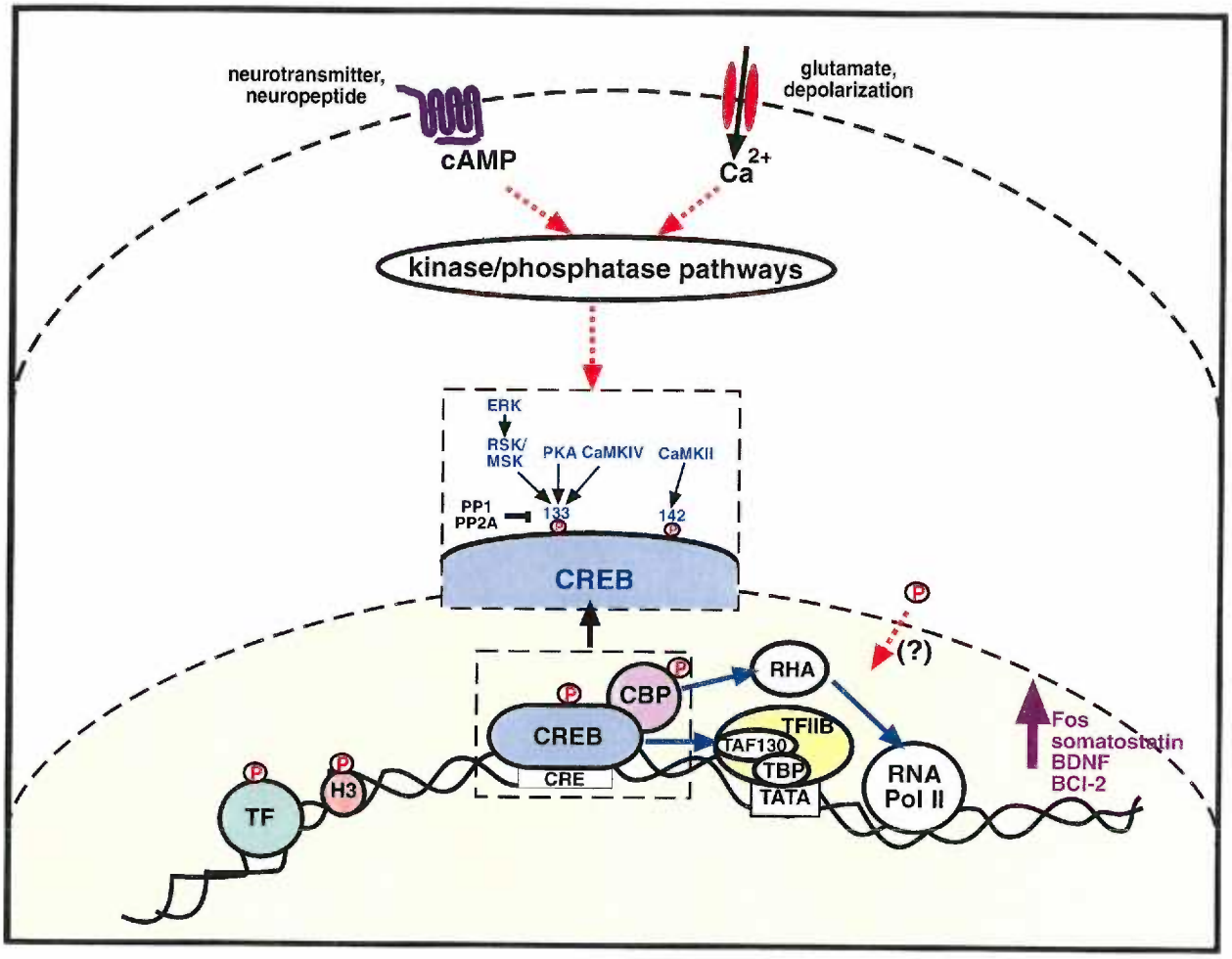

Figure 1.3. **CREB regulation by calcium and cAMP.** Both calcium and cAMP can regulate CREB-dependent transcription through variety of regulatory pathways. The best described mechanism is the phosphorylation of CREB. The phosphorylation state of CREB can be controlled by a number of different kinases and phosphatases. Other targets for the regulation of CREB-dependent transcription include the coactivator, CBP. In addition, calcium and cAMP can regulate the phosphorylation of other transcription factors (TF) and histones (H3), events that are often required for CRE-dependent transcription. Finally, the activity of components of the basal transcriptional machinery may also be regulated by either calcium- or cAMP-induced phosphorylation. The net effect of these signaling events is the up regulation of CREB-dependent target genes such as Fos, somatostatin, BDNF and Bcl-2.

by recruitment to the membrane and subsequent phosphorylation (Morrison and Cutler, 1997). Upon activation, Raf kinases can then phosphorylate and stimulate the MEK kinase family members, MEK 1 and 2(Dent, et al., 1992; Lange-Carter, et al., 1993; Morrison and Cutler, 1997). The MEKs are a unique class of enzyme capable of phosphorylating both tyrosine and serine/threonine residues. The only well-described downstream targets of the MEK kinases are ERKs (Crews, et al., 1992; Crews and Erikson, 1993; Schaeffer and Weber, 1999; Zheng and Guan, 1993; Zheng and Guan, 1993). There are currently five members of the ERK family, of which ERKs 1 and 2 have been most extensively studied. Activation of ERKs involves the requisite phosphorylation of two tyrosine and threonine residues by MEKs (Canagarajah, et al., 1997). These phosphorylations induce a conformational change in ERK structure, exposing the kinase domain and allowing access to ERK substrates (Canagarajah, et al., 1997). The ERKs are proline-directed kinases and recent biochemical and crystallographic studies have begun to characterize the molecular bases for both ERK substrate recognition and phosphorylation (Canagarajah, et al., 1997; Holland and Cooper, 1999). Activated ERKs are capable of phosphorylating many cytoplasmic and membrane-associated proteins. In addition, upon stimulation ERKs are able to dimerize and translocate into the nucleus (Khokhlatchev, et al., 1998). Here they can phosphorylate a variety of proteins including a number of transcription factors (see chapter two). These nuclear actions of ERKs underlie their ability to regulate gene expression in many cell types. Within neurons, ERKs are capable of mediating both rapid and slow changes in neuronal function (Sweatt, 2001). The rapid actions, in many cases involve the direct phosphorylation of ion channels (e.g. Kv4.2 potassium channel) and other signaling kinases (e.g. RSKs, MSKs) (Adams, et al., 2000). In contrast, the ability of ERKs to regulate transcription may underlie their slower, long-term effects. Over recent years, accumulating evidence has implicated ERKs in the regulation of a number of neuronal functions including differentiation (Marshall, 1995), synaptic plasticity (Impey, et al., 1999) and cell survival (Goldberg and Barres, 2000). In chapter two, we provide a

detailed discussion of recent advances in our understanding of both the regulation and actions of ERK signaling in neurons.

In addition to the core three enzyme module, other proteins can regulate ERK signaling. These include a number of kinases that phosphorylate and control the activity state of components of the RAF-MEK-ERK module. A notable example is the stimulation of Raf-1 (Morrison and Cutler, 1997). Recent studies have revealed a complex picture in which a number of tyrosine or serine/threonine kinases are able to phosphorylate Raf-1 and either potentiate or inhibit its kinase activity (King, et al., 1998; Mason, et al., 1999; Rommel, et al., 1999; Zimmermann and Moelling, 1999). In addition, a number of scaffold molecules can interact with the RAF-MEK-ERK signaling module. For example, 14-3-3 is a protein that binds to specific phosphorylated serine residues on Raf-1 and regulates its activation state (Fantl, et al., 1994; Tzivion, et al., 1998). Furthermore, proteins like KSR and MP-1 can specifically interact with multiple components within the ERK signaling cascade (Downward, 1995; Schaeffer, et al., 1998). While the function of these molecules is not entirely clear, they probably serve a scaffolding role to ensure specific and appropriate activation of ERKs in response to distinct signals. Ultimately, it is likely that while the activation of the RAF-MEK-ERK module is central to the stimulation of ERK signaling, the coordinated action of numerous other kinases and scaffold molecules is important in controlling ERK signal specificity.

An important consideration is the mechanism by which this RAF-MEK-ERK signaling unit is activated. As discussed, the initial event in Raf activation involves recruitment of the enzyme to the membrane. Research over the last decade has indicated that the Ras family of small G-proteins are important mediators of Raf activation (Burgering and Bos, 1995). Activation of Ras is central to the regulation of ERKs by a variety of extracellular stimuli, as discussed below. However, recent evidence from our laboratory has identified the Ras-

related G-protein, Rap1, as an additional regulator of ERKs, particularly in neurons (Grewal, et al., 1999). A central goal of this thesis is to further our understanding of Rap1-dependent signaling.

Ras-dependent signaling

The Ras superfamily of small GTPases can regulate a diverse array of processes such as cell proliferation, cell differentiation and apoptosis (Lowy and Willumsen, 1993; Maruta and Burgess, 1994). Not surprisingly, mutations in Ras are associated with a variety of cancers (Lowy and Willumsen, 1993). Like all small G-proteins, the Ras GTPases are active in the GTP-bound state and inactive in the GDP bound state. Various studies have identified a number of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) that regulate the activation state of Ras proteins (Burgering and Bos, 1995). These GEFs and GAPs are targets of numerous extracellular signals including growth factors, hormones and neurotransmitters and, as such, the Ras family represent major players in signaling through these various stimuli (Lowy and Willumsen, 1993). For example, stimulation of growth factor receptor-tyrosine kinases leads to the autophosphorylation of their intracellular domains and subsequent recruitment of various 'adapter' molecules such as SHC and FRS2 (see chapter 2) (Schlessinger, 1993; Schlessinger, 1994; Schlessinger and Bar-Sagi, 1994). These adapter molecules are important mediators of protein-protein interactions. As such they can bind to and induce the re-localization of molecules such as SOS, a Ras GEF, and RasGAP, a GAP protein, to the membrane where they can regulate Ras activity (Schlessinger, 1993). This mode of Ras activation via growth factor signaling is a highly conserved mechanism that has been described both in mammalian systems (Schlessinger, 1993) and genetic systems such as *Drosophila* (Duffy and Perrimon, 1996; Duffy and Perrimon, 1994) and *C elegans* (Sundaram and Han, 1996)

Activation of Ras can lead to the stimulation of numerous signal transduction pathways. For example, the PI3 kinase signaling pathway can be activated in a Ras-dependent manner (Rodriguez-Viciana, et al., 1994). Moreover, other small G-proteins, particularly those of the Rac/Rho/CDC42 family, are downstream of Ras signaling (Bar-Sagi and Hall, 2000). However, the best described target for Ras signaling is RAF. Ras is membrane-anchored by virtue of post-translational C-terminal lipid modifications (Hancock, et al., 1989; Hancock, et al., 1990). Upon activation, Ras is able to bind to and recruit Raf to the membrane (Vojtek, et al., 1993). Here, Raf becomes phosphorylated through poorly understood mechanisms (Morrison and Cutler, 1997). Membrane recruitment and phosphorylation of Raf is sufficient for its full activation and subsequently allows stimulation of MEKs and ERKs (see Fig 2.1).

The Ras-dependent cascade is perhaps the most well studied ERK signaling pathway. In mammalian cells, it can mediate the actions of many growth factors on cell proliferation and differentiation (Kerkhoff and Rapp, 1998). In addition, it can regulate a number of developmental programs including eye development in *Drosophila* (Duffy and Perrimon, 1994) and vulva induction in *C elegans* (Sundaram and Han, 1996). As such, the Ras-ERK kinase cascade represents a versatile and evolutionarily conserved signaling pathway. However, recent studies have begun to identify Ras-independent mechanisms by which ERKs become activated. One pathway that is a central focus of this thesis is the Rap1–dependent pathway described below.

Rap1, a regulator of ERK signaling

There are four members of the Rap family of small G-proteins, Rap1A and B, and Rap2A and B(Bos, 1998). Rap1A and B exhibit a very high degree of structural homology and

have been the best studied of the Rap proteins (Bos, 1998). Rap1 was originally identified and characterized in Drosophila and mammals as an antagonist of Ras signaling (Kitayama, et al., 1989; Kitayama, et al., 1989). For example, in fibroblast cells, Rap1 expression could prevent oncogenic Ras-induced transformation (Kitayama, et al., 1989; Kitayama, et al., 1989). These inhibitory actions have been attributed to the ability of Rap1 to block Ras-mediated stimulation of ERKs (Cook, et al., 1993; Vossler, et al., 1997). This inhibition seems to occur at the level of Raf1 stimulation, since Ras activity is not affected . The mechanism is still unclear but may involve the ability of Rap1 to bind to but not activate Raf-1, thus sequestering it away from Ras. The physiological significance of this inhibitory effect of Rap1 on ERK signaling remains to be fully appreciated. Nevertheless, it has been suggested that Rap1 activation may be important in regulating T-cell receptor signaling to ERKs following antigenic stimulation of lymphocytes (Boussiotis, et al., 1997). In addition, the ability of cAMP to stimulate Rap1 has been suggested to underlie its ability to antagonize growth factor stimulation of ERKs and subsequent proliferation in various cell types (Vossler, et al., 1997). However, it should be noted that some reports have questioned the ability of Rap1 to inhibit ERK signaling (Zwartkruis, et al., 1998).

Recent studies in our laboratory have identified a pathway by which Rap1 can stimulate, rather than inhibit, ERK signaling. This pathway depends on the expression of the Raf isoform, B-Raf. Unlike Raf1, Rap1 is able to activate B-Raf (Vossler, et al., 1997). This activation is analogous to Ras signaling, where GTP-bound Rap1 is able to bind to and recruit B-Raf to the membrane where it becomes fully stimulated (Vossler, et al., 1997). Active B-Raf is then able to stimulate MEK and ERKs. This Rap1:B-Raf pathway was originally characterized in the context of cAMP signaling in PC12 cells.(Vossler, et al., 1997), a pheochromocytochroma cell line that has provided a useful model of neuronal signaling. These cells contain high levels of B-Raf, such that the PKA-dependent activation of Rap1 by cAMP allowed it to stimulate ERK signaling. One significance of

these findings lies in the fact that this Rap1-dependent pathway may explain the cell-type specific effects of cAMP on growth factor signaling. In B-Raf deficient cells, cAMP activation of Rap1 can lead to inhibition of Ras-signaling. In many cases this blocks the actions of growth factors. In contrast, in the presence of B-Raf, cAMP can now positively couple to ERK signaling and potentiate growth factor actions (Vossler, et al., 1997).

Rap1 can also be activated by certain growth factors, and this may explain the generation of growth factor signaling specificity (York, et al., 1998). For example, in PC12 cells, two growth factors, nerve growth factor (NGF) and epidermal growth factor (EGF) both signal via ERKs yet they induce distinct physiological responses. NGF stimulates differentiation into a sympathetic neuron-like phenotype, while EGF stimulates proliferation. It is thought that these distinct actions are controlled by the duration of ERK signaling elicited by both growth factors. Studies in our laboratory have demonstrated that the ability of NGF to activate both Ras- and Rap-dependent pathways in PC12 cells allows it to induce a sustained activation of ERKs and thus stimulate neuronal differentiation (York, et al., 1998). In contrast, growth factors such as EGF, which only activate Ras, induce transient ERK activation and subsequently induce cell proliferation.

Both Rap1 and B-Raf are highly expressed within the nervous system. This suggests that the Rap1:B-Raf pathway may be an important stimulator of ERK signaling during the regulation of neuronal function. In addition, given our findings in PC12 cells, it may provide an alternate route to the Ras-dependent pathway in the activation of ERKs by various stimuli, such as neuronal growth factors and neurotransmitters that elevate cAMP levels. For example, recent studies have demonstrated that activation of ERKs may underlie the ability of cAMP to regulate processes such as neuronal survival in cerebellar granule cells (Villalba, et al., 1997) and synaptic plasticity both in *Aplysia* sensory neurons (Martin, et al., 1997) and rat hippocampal slices (Winder, et al., 1999). While these

actions are consistent with activation of a Rap1:B-Raf signaling pathway, the regulation and role of this pathway in neuronal signaling has not been examined. Therefore, the central goal of this thesis is to further our understanding of the Rap1:B-Raf pathway. These studies have been predominantly carried out in the PC12 cell model of neuronal signaling. In chapter three, we demonstrate that, in addition to cAMP and NGF, stimulation of calcium influx through voltage-operated calcium channels can also activate the Rap1:B-Raf pathway leading to ERK activation. In chapter four, we show that the Rap1-ERK pathway is required for the activation of CREB-dependent transcription by both calcium and cAMP signals in PC12 cells.

Thesis Aims

The major focus of this thesis is to test the following:

1) Is activation of the Rap1:B-Raf pathway a target for calcium signaling to ERKs ?

As discussed, calcium signaling can use several signaling pathways including PKA or ERK to regulate neuronal function. Given our previous findings with cAMP and NGF signaling, it is possible that some of the actions of calcium will reflect signaling through a PKA-dependent Rap1-ERK pathway. This hypothesis will be examined in PC12 cells. This cell line has proved invaluable in the study of neuronal signaling pathways. In particular, these cells express high levels of voltage-gated L-type calcium channels. Thus, upon membrane depolarization of the cells by application of high KCl concentrations, calcium influx can be induced. This provides a simple way to examine calcium-mediated signal transduction pathways.

2) Can the Rap1-ERK pathway regulate CREB-dependent transcription ?

The activity of CREB can be regulated by both PKA and ERK signaling pathways. Some of this regulation may involve cross-talk between PKA and ERK cascades. The role of Rap1 signaling in the regulation of CREB-dependent transcription will therefore be examined. These studies will also be performed in PC12 cells, in the context of both cAMP and calcium signaling.

CHAPTER TWO

Extracellular signal-regulated kinase signalling in neurons.

SAVRAJ S. GREWAL[‡§], RANDALL D YORK[‡],

PHILIP J. S. STORK[‡§]

[‡]Vollum Institute for Advanced Biomedical Research, L474, and [§]Department of Cell and Developmental Biology,

Published in *Current Opinion in Neurobiology* (1999) **9**, 544-553

Summary

Extracellular signal-regulated kinases (ERKs) are emerging as important regulators of neuronal function. Recent advances have increased our understanding of ERK signalling at the molecular level. In particular, it has become evident that multiple second messengers such as cyclic-adenosine monophosphate, protein kinase A, calcium and diacylglycerol, can control ERK signalling via the small G-proteins, Ras and Rap1. These findings may explain the role of ERKs in the regulation of activity-dependent neuronal events such as synaptic plasticity, long-term potentiation and cell survival. Moreover, they allow us to begin to develop a model to understand both the control of ERKs at the subcellular level as well as the generation of ERK signal specificity.

Introduction

The mitogen activated protein/extracellular signal-regulated kinases (MAP kinases or ERKs) regulate a diverse array of functions such as cell growth and proliferation, differentiation and apoptosis (Schaeffer and Weber, 1999). Such actions have been best examined in the context of growth factor signalling via receptor tyrosine kinases (RTKs) (van der Geer, et al., 1994). These studies, predominantly carried out in non-neuronal cell lines, have characterized what could be considered as the archetypal ERK cascade involving the activation of the small G-protein, Ras, and the kinases, Raf-1 and MEK (Figure 2.1). The role of ERKs in the regulation of neuronal function has received increasing attention. While much of this work has focused on the neurotrophic actions of neuronal growth factors (Segal and Greenberg, 1996), two emerging themes have developed over the past few years. Firstly, it has become apparent that ERK signalling pathways can play multiple roles in the activity-dependent regulation of neuronal function.

Secondly, a number of studies have identified novel pathways involving the integration of 2^{nd} messenger systems such as Ca^{2+}, cyclic-adenosine monophosphate (cAMP)/ protein kinase A (PKA) and diacylglycerol (DAG) in the regulation of ERK signalling. These themes highlight issues unique to the understanding of signal transduction mechanisms in neurons. In the present review we will outline these new developments and attempt to develop a model for ERK action in neurons.

Novel ERK signalling mechanisms

PKA and Rap: Positive regulators of neuronal ERKs

The examination of the actions of NGF in the PC12 cell line has provided important information about neurotrophin-mediated signalling pathways. In these cells, NGF treatment induces differentiation into a sympathetic neuron-like phenotype (Greene and Tischler, 1976). This effect is associated with sustained activation of ERKs and a role for a Ras-dependent pathway has been well established (Marshall, 1995). Recent studies have identified a novel mechanism for NGF-induced stimulation of ERKs (Figure 2.1). This pathway involves the PKA-dependent activation of the Ras-related small G-protein, Rap1, and the Raf isoform, B-Raf (Yao, et al., 1998; York, et al., 1998). Given that B-Raf is highly localized to neuronal and neuroendocrine cells, this Rap1-dependent pathway may be unique to these populations. Indeed, the ability of PKA and Rap1 to couple to ERK activation may have significant implications for neuronal signalling. Inhibition of Rap1 function in PC12 cells blocked features of neuronal differentiation such as induction of gene expression and sodium channel function. Moreover, recent studies in *Drosophila* have further emphasized the role of both the Rap1 activator, C3G, and Rap/Ras in neuronal determination (Ishimaru, et al., 1999). Finally, PKA stimulation of ERK activity may also

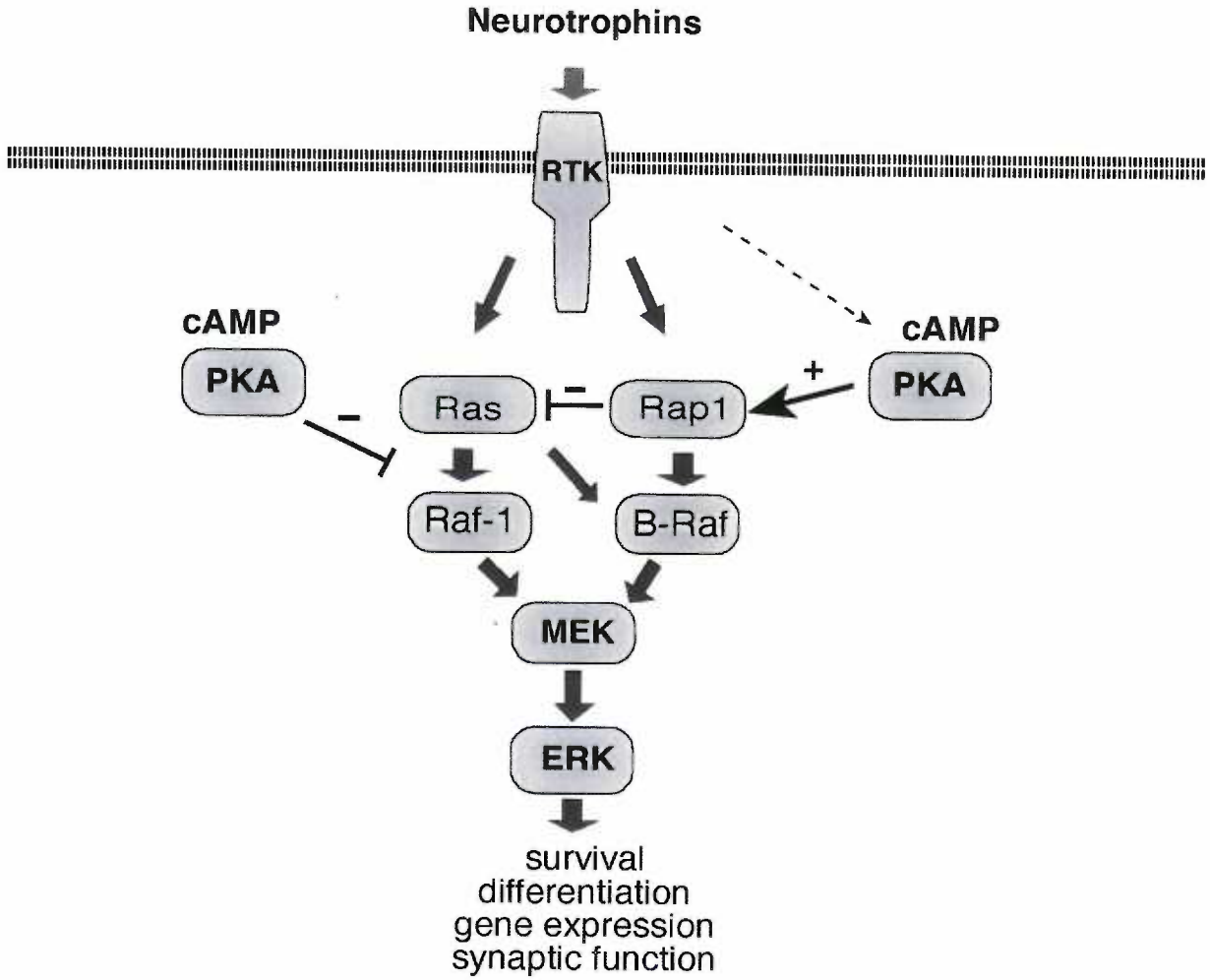

Figure 2.1: **Ras- and Rap-dependent ERK signalling cascades.**

Growth factors can signal to ERKs via the small G-proteins, Ras and Rap1. The Ras-dependent pathway is a ubiquitous signalling cascade, functioning in both neuronal and non-neuronal cell lines. The predominant action of cAMP on this pathway is inhibitory, possibly via the PKA-dependent phosphorylation of Raf-1. In contrast to Ras, the actions of Rap1 are dictated by the expression of B-Raf. In the absence of B-Raf, Rap1 antagonizes Ras-dependent signalling. In the presence of B-Raf, Rap1 can positively couple to ERKs. cAMP can activate Rap1, either by direct stimulation of GEFs or via PKA. Moreover, PKA may also be required for neurotrophin-mediated activation of Rap1.

regulate both neuronal survival and synaptic plasticity (Martin, et al., 1997; Villalba, et al., 1997).

GEFs and GAPs: Regulating Ras and Rap1

Ras and Rap1 activity is tightly regulated by specific guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The last few years have led to significant progress in both the identification and characterization of novel members of these two families as well as a further understanding of the regulation of existing GEFs and GAPs.

In the context of growth factor signalling, the activity of GEFs is generally controlled by their recruitment from the cytoplasm to multi-protein complexes at the cell membrane (Pawson and Scott, 1997). This involves specific SH2-/PTB-domain-containing adaptor molecules that bind to phosphorylated tyrosine residues. For example, SOS and C3G, specific GEFs for Ras and Rap1 respectively, may be recruited to membrane-associated complexes via adaptor molecules such as Grb2 and CrkL. Accumulating evidence suggests that the ability of growth factor receptors to recruit particular adaptor molecules and the specific association of these with either Ras- or Rap-GEFs may provide one level of achieving ERK signal specificity. Our knowledge of GEF regulation has been recently expanded by the identification of new RTK adaptor molecules. For example, upon FGF or NGF stimulation in PC12 cells, FRS2, a membrane-associated molecule, can complex with Grb2 and Shp2, a tyrosine phosphatase (Hadari, et al., 1998). Recruitment of Shp2 appears to be required for the induction of sustained, rather than transient, ERK activity. In addition, upon NGF treatment, FRS2 can also bind Crk, an effect that may contribute to the activation of Rap-mediated pathways during sustained ERK stimulation (Meakin, et al., 1999). More recently, Ginty and co-workers have described two novel molecules, rAPS and SH2-B, that can bind to activated neurotrophin receptors and also lead to ERK

activation via Ras (Qian, et al., 1998). A significant role for all these new adaptor molecules in neuronal function is likely since perturbation of their activity appears not only to affect ERK signalling but also to block processes such as neurite and axonal outgrowth and neuronal viability.

Over the last few years, an expanding area of research has focused on the cross-talk between ERK signalling and other neuronal second-messenger systems. This has been spurred by the identification of novel families of Ras- and Rap-GEFs (Figure 2.2). Interestingly, these GEFs are activated by direct binding to second messengers such as Ca^{2+}, cAMP and DAG (de Rooij, et al., 1998; Ebinu, et al., 1998; Kawasaki, et al., 1998; Kawasaki, et al., 1998). As such, they may represent a new class of GEFs, distinct from the RTK/adaptor molecule-associated GEFs. Furthermore, it is significant to note that they each display very different and restricted CNS expression patterns, suggesting they may act as distinct regulators of neuronal signalling.

GTPase activating proteins (GAPs) mediate the inhibition of Ras and Rap1 signalling. Recently, a novel member of this family, SynGAP, was identified (Chen, et al., 1998; Kim, et al., 1998). This GAP shows a CNS-restricted expression and is specifically localized within post-synaptic densities. Here, it can be inhibited by Ca^{2+} via calcium/calmodulin-dependent (CaM) Kinase II-mediated phosphorylation thus providing an additional mechanism by which neuronal Ca^{2+} may regulate Ras activity.

It is now becoming apparent that both Ras and Rap1 represent important integrators of multiple signalling systems that lead to the activation of ERKs. These signalling pathways appear to be important for neuronal differentiation and determination. Moreover, as discussed below, the identification of novel regulators of ERKs may help expand our understanding of activity-dependent neuronal signalling.

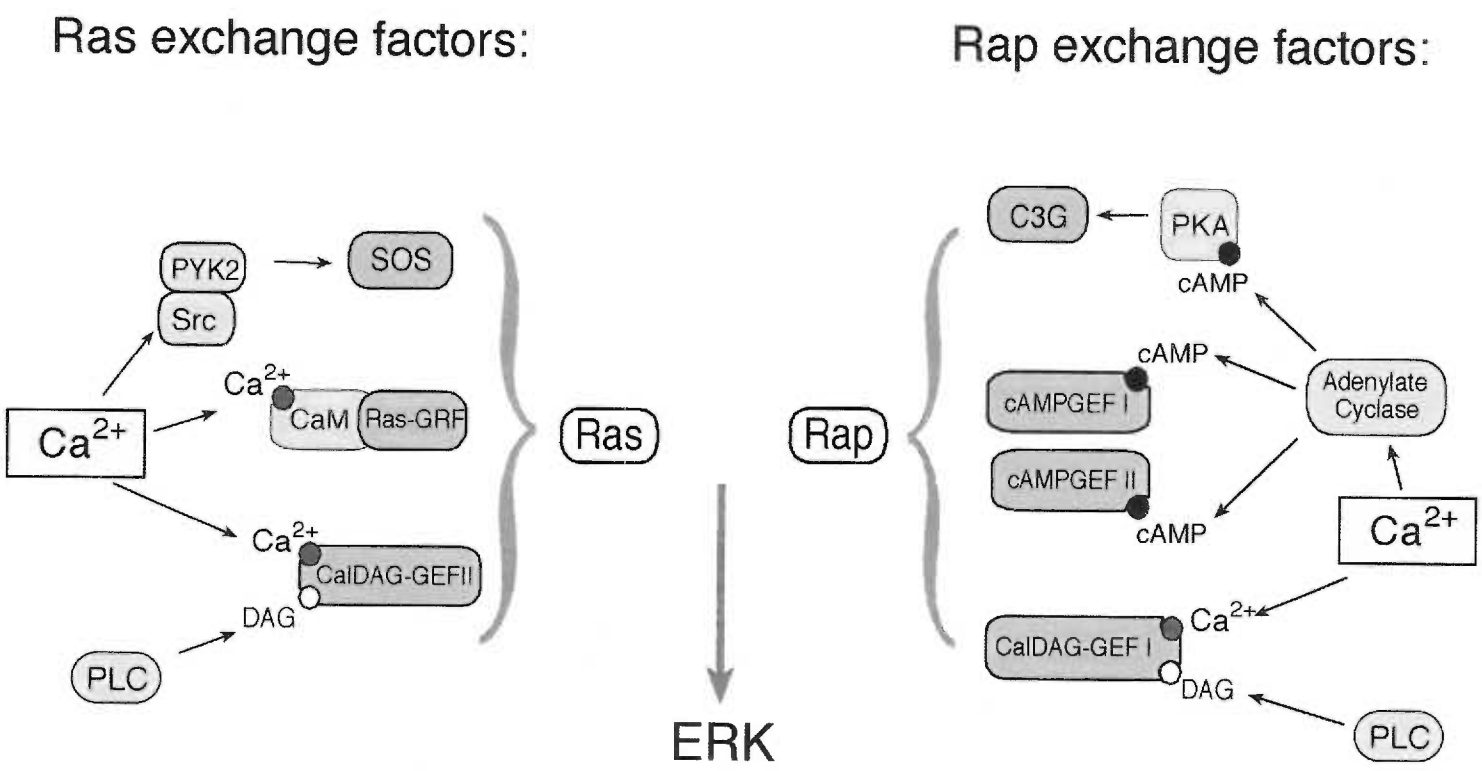

Figure 2.2: **GEF regulation of Ras and Rap1 activity.**

Ras and Rap1 activity can be regulated by multiple GEFs. Perhaps the best studied exchange factors are SOS and C3G. Their activity can be controlled by recruitment to multi-protein complexes at RTKs. Hence they play important roles in growth factor signalling. More recent studies have identified other GEFs that can be regulated directly by intracellular second messengers. For example, CalDAG-GEFII (also called RasGRP) and CalDAG-GEFI can bind directly to and be activated by, both Ca^{2+} and DAG. Likewise, cAMPGEFI (also called Epac) and cAMPGEFII are activate directly by cAMP, independently of PKA. Finally, RasGRF is stimulated by binding to calcium/calmodulin. While the upstream physiological stimuli that control these GEFs are unclear, given their biochemical regulation and discrete CNS expression patterns, they will undoubtedly prove to be important regulators of ERK signalling.

ERKs and activity-dependent neuronal function

Activity-dependent changes in neuronal function are predominantly mediated by elevations in intracellular Ca^{2+} levels. To date, Ras has been widely implicated as a major target for Ca^{2+} signalling to ERKs, particularly through stimulation of a specific GEF, RasGRF (Farnsworth, et al., 1995), or via tyrosine kinases such as Src or Pyk2 (Finkbeiner and Greenberg, 1996). However, given our expanded knowledge about regulation of GEFs and GAPs, it is likely that multiple routes for neuronal activity-dependent stimulation of ERKs exist. For example at excitatory synapses, activity-dependent stimulation of both metabotropic and NMDA/AMPA post-synaptic receptors could lead to elevations in Ca^{2+}, cAMP, PKA and DAG, all of which can potentially regulate both Rap- and Ras-dependent actions. Furthermore, nitric oxide (NO) has recently been reported as an intermediate between NMDA receptor activation and Ras stimulation (Mukherjee, et al., 1999; Yun, et al., 1998). The ability to utilize multiple signalling pathways likely allows neurons to regulate the kinetics of ERK activation. Such mechanisms may be important in translating specific patterns of neuronal activity into qualitatively different intracellular signals (Buonanno and Fields, 1999; Murphy, et al., 1994). Indeed, the ability to temporally regulate Ras- and Rap-dependent signalling has been demonstrated to dictate growth factor effects on gene expression and cell physiology in PC12 cells (York, et al., 1998).

Recent reports have also described the stimulation of ERK signalling pathways *in vivo*. These studies have demonstrated that multiple diverse stimuli such as electroconvulsive seizure (Bhat, et al., 1998), cortico-striatal stimulation (Sgambato, et al., 1998), circadian rhythmicity (Obrietan, et al., 1998), photoreceptor stimulation (Liu, et al., 1998) and opioid withdrawal (Schulz and Hollt, 1998) all can induce selective activation of ERKs

within discrete brain regions. These findings underscore the importance of ERK signalling in the regulation of neuronal function.

Synaptic plasticity, learning and memory and ERKs

Perhaps the most widely examined activity-dependent neuronal process is synaptic plasticity or long-term potentiation (LTP). Accumulating data has identified a prominent role for ERKs in these events. In *Aplysia*, Kandel and colleagues have identified a requirement for ERKs in long-term facilitation (LTF) (Martin, et al., 1997). In particular, LTF stimulation of both ERK activity and nuclear localization could be mimicked by direct stimulation of cAMP. These data may reflect a role for a Rap/B-Raf pathway (Vossler, et al., 1997). ERKs also play a role in mammalian systems. Early studies demonstrated that LTP-inducing stimuli could specifically increase expression of the ERK cascade components, ERK2 and B-Raf, in the hippocampus (Thomas, et al., 1994). More recently, Sweatt and colleagues showed that not only could LTP induce ERK activation but that it was essential for hippocampal LTP induction (English and Sweatt, 1997). This requirement for ERKs may reflect their necessity for cAMP responsive element binding protein (CREB)-mediated gene transcription (Impey, et al., 1998). Additional roles for ERKs in dentate gyrus LTP (Coogan, et al., 1999) and also cerebellar purkinje neuron long-term depression (LTD) (Kawasaki, et al., 1999) have been described. Both LTP and LTD are however multi-faceted phenomena. It is, as yet, unclear whether they reflect changes in pre- or post-synaptic function and the mechanisms that regulate them may vary depending on CNS region examined. Thus, further work is required to provide a detailed explanation for role of ERKs in these phenomena particularly with respect to ERK targets. Current evidence suggests that these may be both nuclear (e.g. CREB-dependent transcription (Impey, et al., 1998)) or cytoplasmic (e.g. ApCAM (Bailey, et al., 1997)). Likely other targets include modulation of ion channel function (Anderson, et al., 1998)

and direct activation of the cell protein synthesis machinery (Lin, et al., 1994; Waskiewicz, et al., 1999). In addition, ERKs can directly phosphorylate synapsin I (Jovanovic, et al., 1996). Given that both Rap1 and the Rap-specific GEF, CalDAGGEFI, are localized at pre-synaptic sites (Kawasaki, et al., 1998; Kim, et al., 1990), this action may represent a mechanism by which neurotrophins exert rapid effects on neurotransmission.

LTP is thought to represent the molecular mechanism that underlies learning and memory. As such, emerging data over the last year, from a number of different model systems, have identified a role for ERKs. For example, mice with a targeted deletion of Ras-GRF exhibit both behavioral deficits in learning paradigms and impaired LTP within the amygdala (Brambilla, et al., 1997). While it is possible that these results reflect loss of Ras signalling to multiple downstream targets, based on the findings described above, a role for ERKs is likely. Furthermore, classic conditioning in *Hermissenda* was shown to be associated with increased ERK phosphorylation (Crow, et al., 1998). More compelling evidence for an ERK-dependent role in learning and memory has come from rodent behavioral studies. In three different models (fear-conditioning, aversive taste learning, and spatial learning) performance was associated with increased ERK activity and inhibition of ERK signalling specifically impaired learning (Atkins, et al., 1998; Berman, et al., 1998; Blum, et al., 1999).

Taken together, these studies provide strong evidence that ERKs are important components of activity-dependent signalling cascades within neurons and that modulation of their activity may be required for both synaptic plasticity, and learning and memory. The generation of mice with targeted disruption in specific components of ERK signalling cascades (e.g. Ras, Rap, GEFs/GAPS, Raf isoforms) should provide further clarification of the mechanisms involved in regulating neuronal function.

Neuronal cell death and ERKs

Neuronal survival can be regulated by both neuronal growth factors and neuronal activity, often in a synergistic manner (Meyer-Franke, et al., 1995). In mammalian systems, an anti-apoptotic role for ERKs in neurons has been proposed and recent studies show that ERKs can protect certain populations of neurons against specific insults (Anderson and Tolkovsky, 1999; Meyer-Franke, et al., 1998). However, perhaps the most interesting recent data linking ERKs with neuronal survival has come from the examination of apoptosis within photoreceptor cells in *Drosophila*. Here, genetic analysis has demonstrated that activation of a Ras-dependent pathway leads to the inactivation of the pro-apoptotic molecule Hid. This occurs both by direct ERK-mediated phosphorylation of Hid and also ERK-dependent down-regulation of Hid expression (Bergmann, et al., 1998; Kurada and White, 1998). Whether such a pathway is conserved in mammalian neuronal systems remains to be determined. In contrast to these anti-apoptotic actions of ERKs, two recent reports suggest that sustained overstimulation of ERK signalling cascades may actually promote neuronal necrotic cell death. (Murray, et al., 1998; Runden, et al., 1998)

Subcellular localization of ERK activation and function

Neurons are perhaps the most polarized cell-type. Dendrites, axons, terminals and cell bodies all exist as functionally specialized regions of the cell. This polarization has unique implications for the understanding of neuronal signal transduction. It is likely that both the stimulation of ERKs as well as their downstream actions will vary based on subcellular localization. As such, the discrete targeting, activation, translocation and the subsequent stimulation of specific targets, may all represent different levels of regulation of ERK signalling specificity in neurons. Recent studies have begun to point to the mechanisms by

which this subcellular regulation may be achieved, particular with resepct to post-synaptic signalling and nuclear actions of ERKs.

Nuclear actions

A major role for ERKs lies in the regulation of gene expression. For example, phosphorylation and activation of the transcription factor CREB can occur in an ERK-dependent manner via stimulation of the CREB kinase RSK2 (Xing, et al., 1996). This action is a major target of both neurotrophin and neuronal Ca^{2+}. Interestingly, PKA has also been shown to utilize an ERK-dependent pathway to CREB (Roberson, et al., 1999). In addition, recent studies have described an action of ERKs downstream of CREB phosphorylation on the co-activator, CBP (Liu, et al., 1998). Thus, ERKs likely have multiple roles in the stimulation of transcription. In many instances, a pre-requisite step for these actions involves the translocation of ERKs from cytoplasm to the nucleus where they can directly phosphorylate and stimulate specific transcription factors (Brunet, et al., 1999). Regulation of this translocation may constitute one level of control of ERK signalling. Indeed a nuclear, but not cytoplasmically, localized constitutively active form of ERK was shown to stimulate the transcription factor, Elk-1, and induce neurite outgrowth in PC12 cells (Robinson, et al., 1998). The mechanisms leading to ERK nuclear localization are still unclear, but do appear to require both ERK phosphorylation and dimerization (Khokhlatchev, et al., 1998). They additionally may depend on the synthesis of nuclear retention proteins (Lenormand, et al., 1998). In yeast, nuclear translocation of the MAP kinase, HOG1, required interaction with a specific isoform of the importin family (Ferrigno, et al., 1998). Interestingly, Storm and co-workers recently demonstrated that, in both PC12 cells and hippocampal neurons, the depolarization-induced nuclear translocation of ERKs and their downstream kinase target, RSK2, utilized a PKA-mediated step (Impey, et al., 1998). The exact mechanism is not certain, although it is interesting to

note that PKA phosphorylation of Dorsal promotes interaction with importin during its nuclear relocalization (Briggs, et al., 1998).

Post-synaptic actions

The post-synaptic density (PSD) represents a functionally specialized region of the neuron involved in the transduction of synaptic transmission into intracellular signalling events. This is primarily achieved by the clustering or anchoring of specific proteins at this region (Kim and Huganir, 1999). Recent studies have identified a number of these molecules, particularly at glutamatergic synapses, that may contribute to the regulation of ERK signalling (Figure 2.3). For example, SynGAP and RasGRF, are specifically enriched at the PSD where they likely regulate Ras activation . Whether Rap1 GEFs/GAPs also localize to the PSD is unknown. Other molecules of interest include AKAP79, which anchors PKA at the PSD (Carr, et al., 1992), and neuronal NOS (nNOS) which associates directly with the scaffold/anchor protein, PSD-95 (Brenman, et al., 1996). This specific localization may allow both molecules to coordinate post-synaptic PKA- and NO-dependent stimulation of Rap1 and Ras pathways respectively.

The Src-like family of tyrosine kinases (e.g. Src, Lyn, Fyn) are emerging as important regulators of post-synaptic signalling. While major PSD targets for these kinases are thought to be AMPA and NMDA glutamate receptors, modulation of ERK activity may also be a consequence of their post-synaptic activation . It is known that Src activation promotes signalling, in part through Ras/Raf-1(Marais, et al., 1995), while Fyn may be an upstream activator of Ras- and Rap-dependent pathways (Boussiotis, et al., 1997). Interestingly, a recent study identified a direct interaction between Lyn and the AMPA receptor (Hayashi, et al., 1999). Here, Lyn appeared to be required for AMPA receptor-mediated activation of ERKs. The mechanism by which Src-family tyrosine kinases

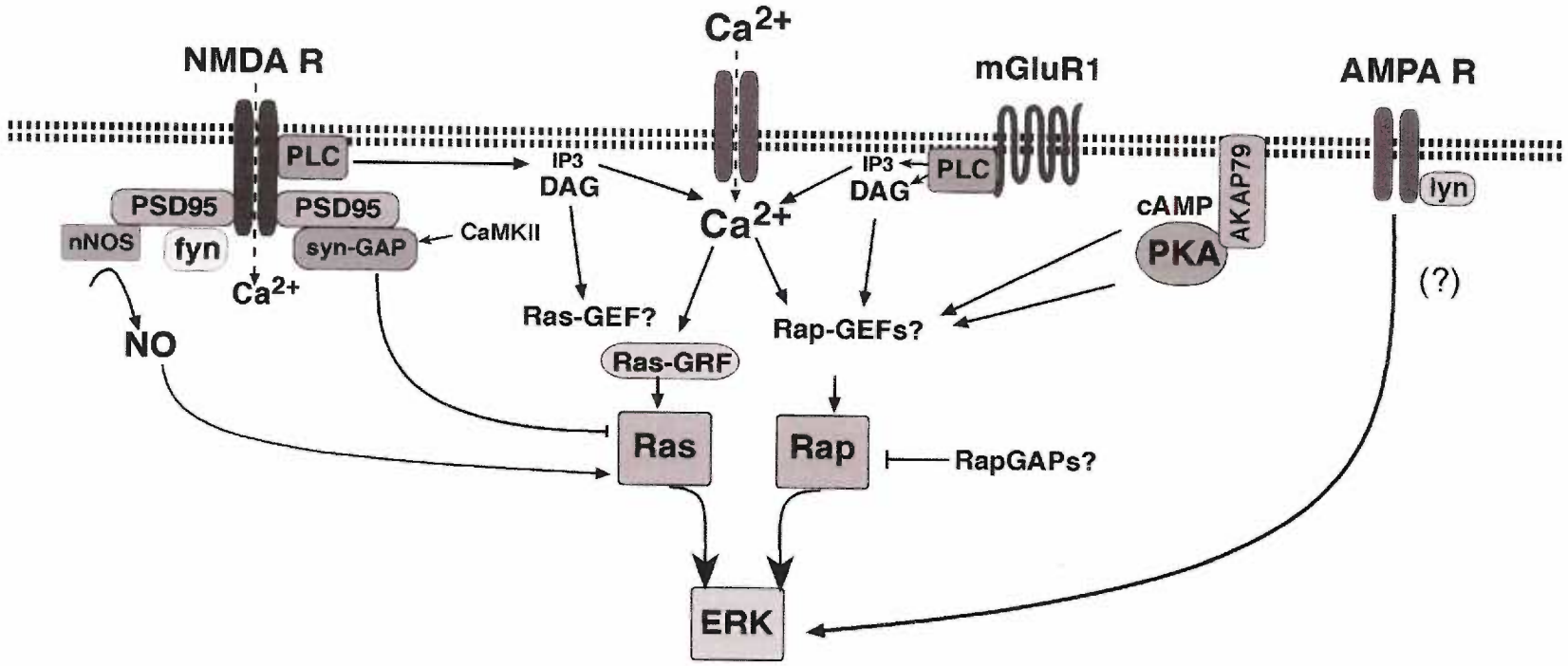

Figure 2.3: **Model of post-synaptic ERK signalling.**

The glutamatergic synapse provides a good model for our understanding of post-synaptic signalling. Numerous studies have demonstrated the anchoring or localization of distinct proteins at these sites. This figure illustrates molecules that may potentially co-ordinate ERK signalling in response to glutamate. Proteins that may function in these pathways but have not yet been identified within the PSD are indicated with a question mark. In this model, a central mediator of these responses is elevated Ca^{2+} levels, either as a result of influx via NMDA channels, or mGluR-induced release of intracellular Ca^{2+} stores. The presence of all these mediators at the PSD (see text) provides the potential for diverse routes to ERK activation in response to different patterns or thresholds of activity-dependent glutamate signalling.

modulate ERK signalling post-synaptically remains to be determined. However, one potentially interesting route may involve their phosphorylation of NMDA and AMPA receptors. Thus, as well as augmenting channel function, tyrosine phosphorylation may also provide anchoring sites for SH2/PTB domain containing adaptor molecules (e.g. Grb2, Shc, Crk) leading to activation of ERK signalling cascades. While such a model still requires experimental validation, it is interesting to note that PLCg can directly associate with phospho-tyrosine sites on NMDA channels via its SH2 domain (Gurd and Bissoon, 1997).

Together, these recent data point to a model of post-synaptic signalling in which ERKs can be stimulated in a number of ways. Whether these pathways reflect synapse-specific signalling mechanisms remains to be determined. Alternatively, the availability of multiple post-synaptic mechanisms of ERK activation may dictate downstream events, e.g. stimulation of ERK pathways to the nucleus versus local ERK-dependent phosphorylation.

Signal propagation and targeting of ERKs

The highly polarized nature of neurons presents interesting problems for ERK signal propagation. For example, if ERK signalling cascades are activated either at nerve terminals or discrete dendritic sites, and their predominant downstream actions are nuclear, how are the signals transduced? In *Aplysia* sensory neurons, serotonin-mediated nuclear translocation of ERKs was suggested to involve relocalization form neuronal processes to the soma . One potential hypothesis to explain ths is that receptor activation promotes internalization and formation of an intracellular signalling vesicle (Grimes, et al., 1997). Indeed, receptor internalization is required for opioid-, serotonergic- and adrenergic-receptor coupling to ERK signalling cascades (Daaka, et al., 1998; Della Rocca, et al.,

1999; Ignatova, et al., 1999). Recent studies using compartmentalized sympathetic neuronal cultures also support this model (Riccio, et al., 1997; Senger and Campenot, 1997). In these systems, discrete activation of neurite terminals by NGF led to internalization and retrograde transport of a NGF-TrkA receptor complex. Importantly, at the cell body, the TrkA receptor was still in the active phosphorylated state, and this activation was required for downstream signalling to CREB (Riccio, et al., 1997).. This 'signalling vesicle' model may explain both nerve-terminal and also postsynaptic, dendritic propagation of ERK signalling to neuronal cell bodies. However, it will be important to determine when and where the various regulatory components of ERK signalling modules become activated. Another related issue concerns the specificity of ERK activation. Given the likely scenario that ERKs can become activated and exert their downstream effects at multiple subcellular locations, how is signal specificity achieved? In the context of PKA signalling, this problem may be solved, in part, by the specific targeting of PKA to precise cellular locations by the AKAP family of anchoring proteins (Pawson and Scott, 1997). Interestingly, recent studies have identified scaffold proteins for both ERK and JNK MAP kinase family members. For example, JIP acts as a scaffold protein for members of the JNK signalling cascade (Whitmarsh, et al., 1998) while MP1 promotes specific complex formation between MEK1 and ERK1 (Schaeffer, et al., 1998). Whether these proteins also promote specific subcellular targeting of ERKs in neurons is not clear. However, we may expect the identification of such regulatory molecules in the near future.

Conclusions

The last few years have demonstrated the potential of ERK signalling cascades to regulate diverse neuronal processes such as cell death, differentiation and synaptic plasticity. At the molecular level this has been realized by the identification of multiple pathways to ERK regulation, particularly via classic second messengers such as cAMP, PKA, DAG and

Ca^{2+}. The next challenge is to begin to address the mechanisms by which these pathways co-ordinate the generation of signal specificity. In addition, future studies aimed at identifying novel ERK targets, particularly at specific subcellular sites, should further our understanding of the role of ERK signalling in neurons.

TABLE 1. Potential neuronal targets of ERK phosphorylation

Nuclear Proteins	
Transcription Factors (TFs)	
ETS family	
Elk1	(Davis, 1993; Price, et al., 1995)
Sap-1	(Price, et al., 1995)
ETS2	(McCarthy, et al., 1997)
ERM, ER81	(Janknecht, 1996; Janknecht, et al., 1996)
ERF	(le Gallic, et al., 1999)
Yan	(Rebay and Rubin, 1995)
PntP2	(Brunner, et al., 1994)
Other TFs	
c-Jun, c-Myc, c-Myb, C/EBPb (NF-IL6)	(Davis, 1993)
Pax6	(Mikkola, et al., 1999)
Stat3, Stat5	(Chung, et al., 1997; Pircher, et al., 1999)
Smad1	(Kretzschmar, et al., 1997)
Other Nuclear Proteins	
Estrogen receptor	(Kato, et al., 1995)
Lamins	(Peter, et al., 1992)
Membrane-Associated Proteins	
plasma membrane	
Epidermal Growth Factor (EGF) receptor	(Takishima, et al., 1991)
Myristoylated alanine-rich C kinase substrate (MARCKS)	(Ohmitsu, et al., 1999)
Connexin 43	(Zhou, et al., 1999)
Aplysia cell adhesion molecule (ApCAM)	(Bailey, et al., 1997)
Phospholipase A2	(Lin, et al., 1993)
cAMP-specific phosphodiesterase (HSPDE4D3)	(Hoffmann, et al., 1999)
vesicle-membrane	
Sypnapsin I	(Jovanovic, et al., 1996)
Caldesmon	(Childs, et al., 1992)
Cytoskeletal Proteins	
Microtubule assoc. proteins (MAP2C, MAP4, Tau)	(Gundersen and Cook, 1999)
Neurofilaments	(Veeranna, et al., 1998)
Myelin Basic Protein	(Erickson, et al., 1990)
Cytoplasmic Kinases	
Rsk2	(Dalby, et al., 1998; Smith, et al., 1999)
RskB	(Pierrat, et al., 1998)
MAPKAP kinase-2	(Stokoe, et al., 1992)
MAPKAP kinase-3 (3pK)	(Ludwig, et al., 1996)
Mnk1/Mnk2	(Fukunaga and Hunter, 1997; Waskiewicz, et al., 1999)
Msk1	(Deak, et al., 1998)
Other Cytoplasmic Proteins	
PHAS-I	(Lin, et al., 1994)
Tyrosine Hydoxylase	(Haycock, et al., 1992)
Hid	(Bergmann, et al., 1998)

CHAPTER THREE

Neuronal Calcium activates a Rap1 and B-Raf Signaling Pathway via the cyclic Adenosine Monophosphate-dependent protein kinase (PKA)

SAVRAJ S. GREWAL[‡§], ANGELA M. HORGAN[‡], RANDALL D. YORK[‡], GINGER S. WITHERS[¶], GARY A. BANKER[¶], AND PHILIP J.S. STORK[‡]

[‡]Vollum Institute, L474, and [§]Department of Cell and Developmental Biology, [¶]Center for Research on Occupational and Environmental Toxicology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201.

Published in *The Journal of Biological Chemistry* (2000) **275**, 3722-3728

Summary

Activity-dependent regulation of neuronal events such as cell survival and synaptic plasticity are controlled by increases in neuronal calcium levels. These actions often involve stimulation of intracellular kinase signaling pathways. For example, the MAP kinase, or ERK, signaling cascade has increasingly been shown to be important for the induction of gene expression and long-term potentiation. However, the mechanisms leading to ERK activation by neuronal calcium are still unclear. In the present study, we describe a PKA-dependent signaling pathway that may link neuronal calcium influx to ERKs via the small G-protein, Rap1, and the neuronal Raf isoform, B-Raf. Thus, in PC12 cells, depolarization-mediated calcium influx led to the activation of B-Raf, but not Raf-1, via PKA. Furthermore, depolarization also induced the PKA-dependent stimulation of Rap1, and led to the formation of a Rap1/B-Raf signaling complex. In contrast, depolarization did not lead to the association of Ras with B-Raf. The major action of PKA-dependent Rap1/B-Raf signaling in neuronal cells is the activation of ERKs. Thus, we further show that, in both PC12 cells and hippocampal neurons, depolarization-induced calcium influx stimulates ERK activity in a PKA-dependent manner. Given the fact that both Rap1 and B-Raf are highly expressed in the central nervous system, we suggest that this signaling pathway may regulate a number of activity-dependent neuronal functions.

Introduction

Activity-dependent changes in neuronal processes such as synaptic plasticity and neuronal survival are mediated in large part through elevations in intracellular calcium levels (Bito, 1998; Franklin and Johnson, 1992; Nicoll and Malenka, 1995). In many cases this involves stimulation of calcium influx, particularly via voltage-operated L-type channels or receptor-operated NMDA channels (Berridge, 1998; Nicoll and Malenka, 1995). Many of the downstream functional consequences of calcium influx involve the regulation of gene transcription (Bito, et al., 1997; Ghosh and Greenberg, 1995; Ginty, 1997). As such numerous studies have begun to examine signal transduction pathways that link neuronal calcium to the regulation of transcription factor activity. These studies have revealed a complexity of calcium-dependent kinase signaling cascades (Ghosh, et al., 1994).

The mitogen activated protein/extracellular signal-regulated kinases (MAP kinases or ERKs) are emerging as important targets for neuronal calcium signaling. ERK activation by neuronal calcium influx has been demonstrated both *in vitro* , using isolated neuronal cultures and the PC12 cell line (Fiore, et al., 1993; Kurino, et al., 1995; Rosen, et al., 1994; Rusanescu, et al., 1995), and *in vivo* after stimulation of neuronal activity (Bhat, et al., 1998; Sgambato, et al., 1998; Vanhoutte, et al., 1999). Changes in synaptic plasticity are also associated with increases in ERK activity (English and Sweatt, 1996; Martin, et al., 1997). In addition, a role for ERK activity has been demonstrated for both hippocampal long-term potentiation (LTP) (Coogan, et al., 1999; English and Sweatt, 1997; Impey, et al., 1998) and cerebellar long-term depression (LTD) (Kawasaki, et al., 1999). These actions may underlie a requirement for ERKs in learning and memory (Atkins, et al., 1998; Berman, et al., 1998; Blum, et al., 1999; Crow, et al., 1998).

A major issue concerns the mechanism by which calcium influx stimulates ERK activity. ERKs have been best examined in the context of growth factor signaling via receptor tyrosine kinases (RTKs) (Schaeffer and Weber, 1999). These studies have identified a ubiquitous ERK cascade involving the stimulation of the small G-protein, Ras, and the kinases, Raf-1 and MEK (Schaeffer and Weber, 1999). Since both growth factors and neuronal calcium often exhibit synergistic actions, it is possible they may share common signaling pathways. Thus, in both *in vitro* neuronal cultures and PC12 cells, it has been demonstrated that depolarization-mediated opening of L-type calcium channels can activate Ras (Farnsworth, et al., 1995; Rosen, et al., 1994). The mechanism by which this occurs is unclear, but may involve Shc phosphorylation, perhaps via transactivation of the EGF receptor (Rosen and Greenberg, 1996; Zwick, et al., 1997), stimulation of the tyrosine kinases Src (Rusanescu, et al., 1995) or Pyk2 (Lev, et al., 1995), or via activation of the calmodulin-sensitive Ras guanine nucleotide exchange factor (GEF), Ras-GRF (Farnsworth, et al., 1995). Moreover, recent studies have also identified a novel Ras GEF that can be activated directly by calcium and that may contribute to neuronal calcium stimulation of ERKs (Ebinu, et al., 1998; Kawasaki, et al., 1998).

Although Ras represents an important target for neuronal calcium activation of ERKs, it is possible that calcium influx may additionally signal via alternate routes that are Ras-independent. We have recently identified a novel pathway regulating ERK activation via nerve growth factor (NGF) and cAMP (Vossler, et al., 1997; Yao, et al., 1998; York, et al., 1998). Unlike, the well studied Ras-dependent ERK cascade, this pathway involves the PKA-dependent stimulation of the Ras-related small G-protein, Rap1 and the downstream kinase, B-Raf (Vossler, et al., 1997). Given the ability of neuronal calcium to increase intracellular cAMP levels and PKA activity via calmodulin-sensitive adenylate cyclases (Xia and Storm, 1997), this pathway may represent an additional target for activity-dependent signaling. Indeed, studies in *Aplysia* have shown that long-term

facilitation (LTF) involves PKA activation of ERKs, consistent with a role for Rap1/B-Raf (Martin, et al., 1997). In addition, the identification of two novel families of Rap1 GEFs that can activated by direct binding to calcium and cAMP respectively also suggest a role for Rap1 in neuronal calcium signaling (de Rooij, et al., 1998; Kawasaki, et al., 1998).

In this study, we examine the possible contribution of Ras-independent signaling pathways to the activation of ERKs by neuronal calcium. Using depolarization-mediated activation of L-type calcium channels in both PC12 cells and hippocampal neurons, we show that calcium influx stimulates ERKs in a PKA-dependent manner. Consistent with these findings, we further demonstrate that depolarization activates both Rap1 and B-Raf via PKA, and also induces formation of a Rap1/B-Raf signaling complex. Rap1 is highly expressed in the CNS and B-Raf is the predominant neuronal Raf isoform. As such we propose that the PKA-dependent activation of a Rap1/B-Raf signaling pathway may represent a novel target for neuronal calcium signaling to ERKs. Taken together with previous findings, we suggest that the ability of neuronal calcium to activate both PKA/Rap1- and also Ras-dependent ERK pathways may be important in the regulation of events such as synaptic plasticity and neuronal survival.

Experimental procedures

Materials - PC12-GR5 cells were kindly provided by R. Nishi, Oregon Health Sciences University, Portland, Oregon. Agarose-conjugated antibodies to ERK1 and ERK2 (c-16), antibodies to B-Raf and Raf-1, and recombinant MEK-1 protein were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Anti-Flag (M2) antibody was purchased from Sigma. Forskolin, KT5720, and N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H89), W12 [N-(4-Aminobutyl)-1-naphthalenesulfonamide, HCl] and W13 [N-(4-Aminobutyl)-5-choloro-1-naphthalenesulfonamide, HCl] were purchased

from CalBiochem (Riverside, CA). Phosphorylation-specific ERK antibodies that recognize phosphorylated ERK1 and ERK2, at residues threonine 183 and tyrosine 185 were purchased from New England Biolabs (Beverly, MA). Nickel agarose (Ni-NTA-Agarose) was purchased from Qiagen Inc. (Chatswoth, CA.). Radioisotopes were from NEN-DuPont. All other reagents were from Sigma (St. Louis, MO).

Cell culture - PC12 cells were maintained in DMEM (Dulbecco-Modified Eagle Medium) plus 10% horse serum and 5% fetal calf serum on 100 mm plates to 50-60% confluence at 37° C in 5% CO_2 prior to harvesting. For immune complex assays and western blotting, cells were maintained in DMEM plus 0.5% horse serum for 16 hours at 37° C in 5% CO_2 prior to treatment with various reagents. All inhibitors were added to plates 20 minutes prior to treatment. Where indicated, PC12 cells were differentiated by culturing in 50 ng/ml NGF for ten days.

Hippocampal cells were harvested from embryonic (E18) rats as described (Goslin and Banker, 1991). Briefly, dispersed hippocampal cells were plated on coverslips, on top of a monolayer of glial cells. Cells were grown for 4-5 days *in vitro* and tetrodotoxin was added 14 hr prior to treatment to decrease basal activity and to eliminate action potential firing during depolarizing treatments. For immunohistochemistry, cover slips containing hippocampal cells were treated as described and fixed in 4% paraformaldehyde/4%sucrose. Subsequent immunohistochemistry was performed using primary phospho-ERK and ERK1/2 antibodies (New England Biolabs, 1:1000) and rhodamine -goat anti rabbit or FITC-goat anti mouse IgG secondary antibodies (1:500). Cells were examined by fluorescent microscopy. The signal intensity within cell bodies was quantified for each treatment condition using NIH-Image and expressed as fold increase over untreated conditions, after subtracting background levels.

Western blotting - Cell lysates were prepared as described (Vossler, et al., 1997). Protein concentrations were assessed using Bradford protein assay. For detection of phospho-ERK1/2, equal protein amounts of cell lysate per treatment condition were resolved by SDS-PAGE, blotted onto PVDF filters and probed with phospho-ERK antibodies as per manufacturers instructions.

Plasmids and Transfections - Fifty percent-confluent PC12 cells were co-transfected with the indicated cDNAs using a calcium phosphate transfection kit (Gibco BRL) according to the manufacturer's instructions. The vector pcDNA3 (Invitrogen Corp.) was added to each set of transfections to ensure that each plate received the same amount of DNA. Four hours following transfection, cells were glycerol shocked and allowed to recover in serum containing media for 24 hr. Cells were then starved overnight in serum free DMEM before treatment and harvest.

Immune complex assays - For ERK assays, treated and untreated cells were lysed in a buffer containing 10% Sucrose, 1% NP-40, 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 1 mg/ml Leupeptin, 1mM sodium orthovanadate, and 10 mM sodium fluoride. The lysates were spun at low speed to remove nuclei and the supernatant was collected. ERK2 was immunoprecipitated from lystates containing equal protein amounts per treatment condition and kinase activity was measured by immune complex assay as described (Vossler, et al., 1997) using [^{32}P]-gATP with myelin basic protein (MBP) as substrate. For Raf assays, untreated and treated cells were lysed in 1% NP-40 buffer containing 10 mM Tris pH 7.4, 5 mM EDTA, 50 mM NaCl, and 1 mM PMSF. The lysates were spun at low speed to remove nuclei and the supernatant was collected. Raf-1 or B-Raf was immunoprecipitated from equal protein amounts of lystate per treatment condition and activity was measured by immune complex assay as described (Vossler, et al., 1997) using [^{32}P]-gATP with MEK as substrate. The reaction

products of all kinase assays were resolved by 10% SDS-polyacrylamide gel and analyzed with a PhosphorImager (Molecular Dynamics).

Nickel affinity chromatography - For studies examining polyhistidine-tagged Rap1, transfections were performed using calcium phosphate. Cells were lysed in a buffer containing 1% NP40, 10mM Tris, pH 8.0, 20 mM NaCl, 30 mM $MgCl_2$, 1mM PMSF, and 0.5mg/ml aprotinin and supernatants prepared. Transfected His-Rap1 proteins were precipitated from supernatants containing equal amounts of protein using Ni-NTA Agarose and washed with 20mM imidazole in lysis buffer and eluted with 500 mM imidazole, 5mM EDTA in Phosphate Buffered Saline. Eluates containing His-tagged proteins were separated on SDS-PAGE and B-Raf protein was detected by western blotting (Vossler, et al., 1997). Equal amounts of each eluate were immunoprecipitated with B-Raf antisera and B-Raf activity measured by immune complex assay. Equal amounts of His-Ras or His-Rap1 in the eluates was confirmed by western blotting.

Rap1-GTP loading assays - PC12 cells were transfected with Flag-tagged Rap1B. Following treatments cells were lysed, Flag-Rap1b immunoprecipitated and GTP-loading assay performed as described previously (Vossler, et al., 1997).

Results

Neuronal depolarization activates ERKs in a PKA-dependent manner. Membrane depolarization of PC12 cells following addition of KCl activated ERK2 in a concentration-dependent manner. (Figure 3.1A). Sixty mM KCl induced a rapid and sustained increase in ERK activity as assessed by both immune-complex kinase assay (Figure 3.1B) and also phospho-specific antibodies (Figure 3.1C), with maximal increases occurring at 5-10 minutes. This increase in ERK activity was abolished by pre-treatment

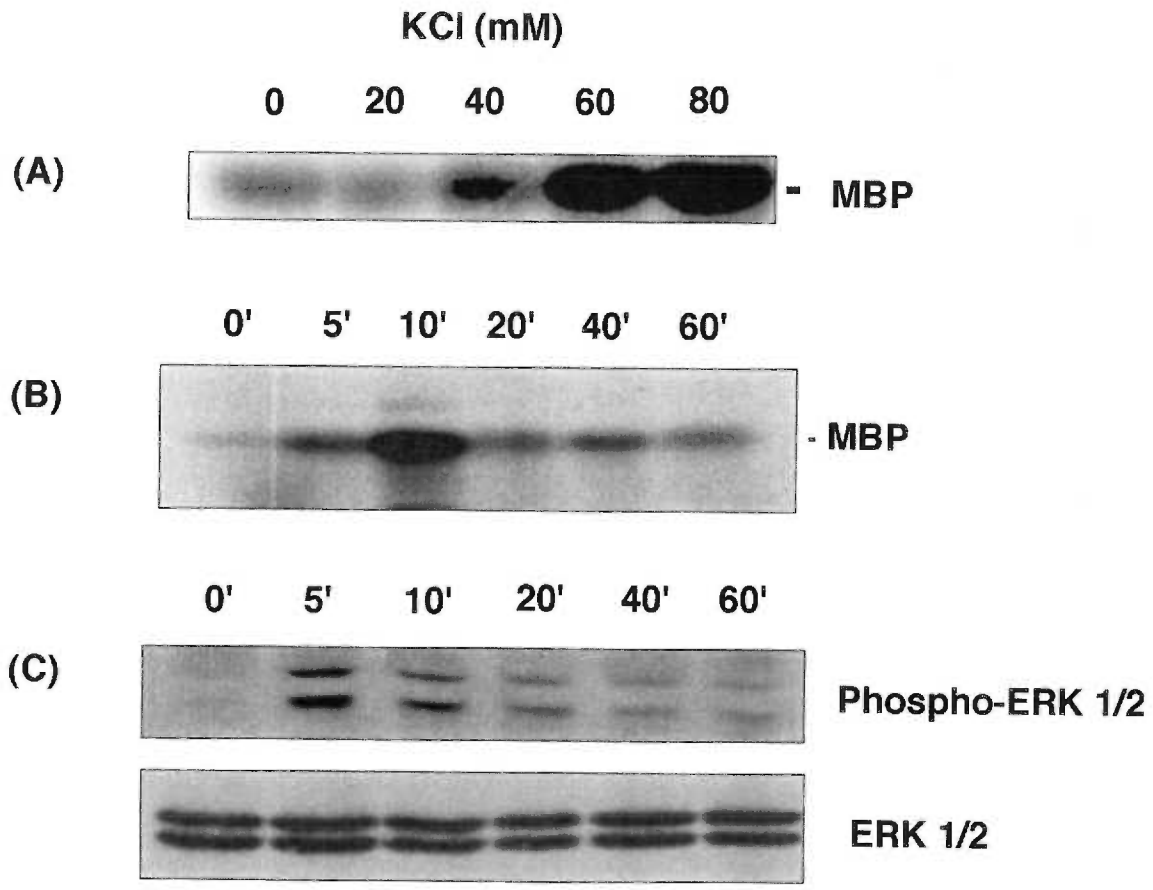

Figure 3.1. **Activation of ERKs following depolarization of PC12 cells.** Treated cells were harvested for either immune complex kinase assay using myelin basic protein (MBP) as a substrate or western blotting using phospho-specific ERK1/2 antibodies. (A) PC12 cells were treated with KCl at the concentrations indicated for 10 min. Immune complex assay with equal protein amounts of cell lysate were performed. A representative autoradiogram with the position of MBP is shown. (B) PC12 cells were treated with KCl (60 mM) for the times indicated. Immune complex assay with equal protein amounts of cell lysate were performed. A representative autoradiogram is shown. (C) PC12 cells were treated with KCl (60 mM) for the times indicated. Western blotting with equal protein amounts of cell lysate was performed. Upper panel: probe with phospho-specific ERK1/2 antibody (Phospho-ERK1/2). Bottom panel: parallel western blot probed with ERK1/2 antibody (ERK1/2).

with the L-Type calcium channel antagonist, nifedipine (5uM), consistent with a role for calcium influx (Figure 3.2A, B). Interestingly, inhibition of PKA, using either H89 (Chijiwa, et al., 1990; Engh, et al., 1996) or KT5720 (Gadbois, et al., 1992) also blocked KCl-induced increase in ERK activity (Figure 3.2A *upper panel*, B, C) and phosphorylation (Figure 3.2A, *lower panel*).. This requirement for PKA in the actions of depolarization is consistent with the possible stimulation of calcium/calmodulin activated adenylate cyclases. To address this, we also examined the requirement of calmodulin for the activation of ERKs by depolarization. Pretreatment with W13, a calmodulin antagonist (Hidaka and Tanaka, 1983) blocked the depolarization-mediated increase in ERK activation (Figure 3.2D). In contrast, W12, a structurally related, inactive analog of W13 (Hidaka and Tanaka, 1983) had no effect. Finally, this PKA-dependent activation of ERKs appeared specific for depolarization: ERK activation by ionomycin, a calcium ionophore, was unaffected by pre-treatment with H89 (Figure 3.2E).

We further examined this requirement for PKA in the activation of ERKs in two other neuronal model systems. We first looked at PC12 cells which had been neuronally differentiated by culturing for ten days in the presence of nerve-growth factor. As with undifferentiated cells, depolarization of neuronal PC12 cells also activated ERKs in a PKA-dependent manner as assessed by H89 inhibition of ERK phosphorylation (Figure 3.3A). We next examined the actions of depolarization in dissociated hippocampal neuron cultures. Using immunofluoresence, we found that depolarization of neurons for ten minutes induced a marked increased in the phosphorylation of ERKs both within the soma and dendrites (Figure 3.3B, C). Interestingly, ERK phosphorylation appeared largely absent from axons (data not shown). Pretreatment with H89 inhibited this activation of ERKs (Figure 3.3B, C). This requirement for PKA in ERK phosphorylation within hippocampal neurons was further confirmed by western blot (Figure 3.3C(iii)). Taken together, these

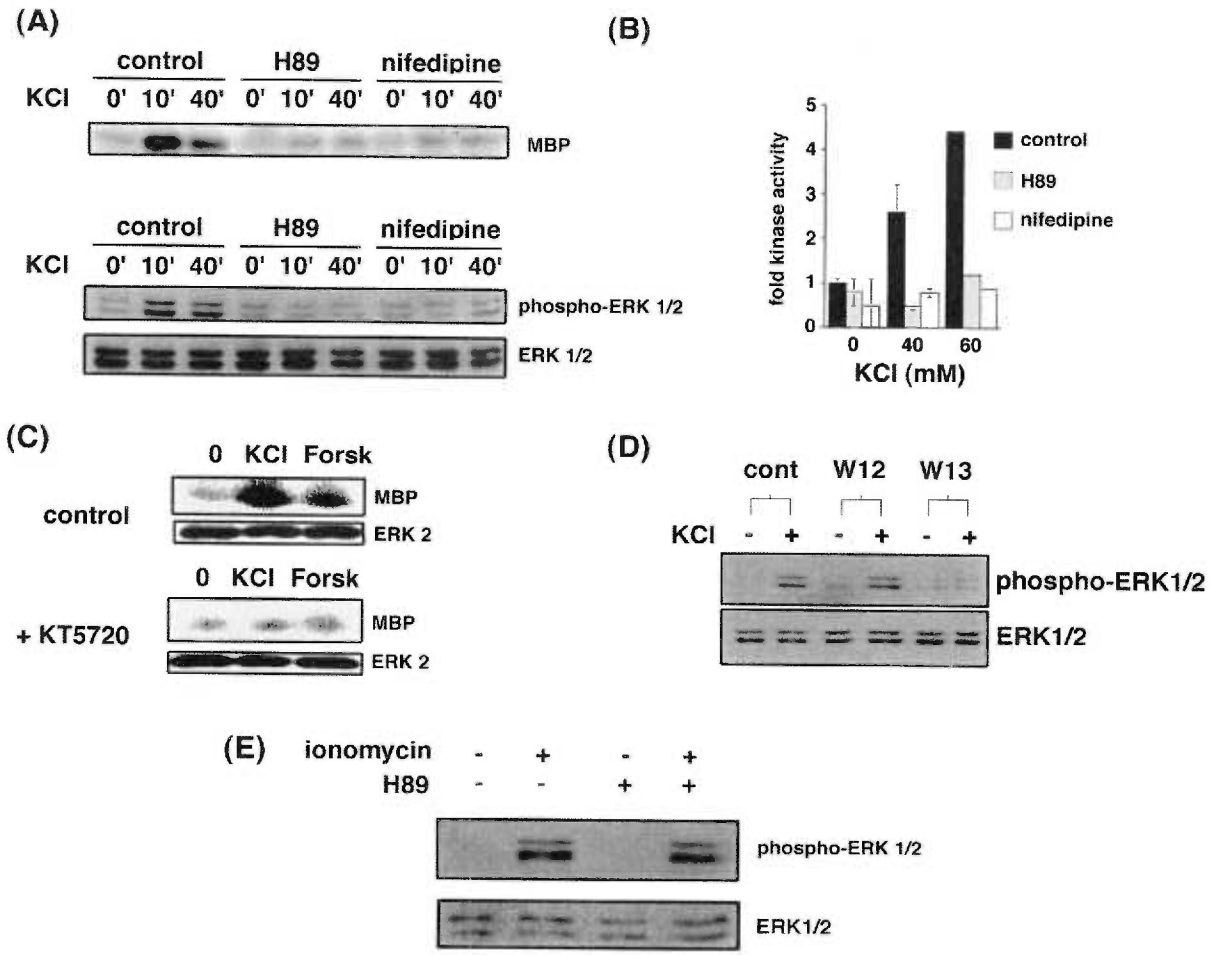

Figure 3.2. **Membrane depolarization activates ERKs via calcium influx, calmodulin and PKA in PC12 cells.** (A) PC12 cells were treated with KCl (60 mM, 10 min) alone, following a pretreatment with H89 (10μM) or nifedipine (5 μM). Equal amounts of cell lysate were assayed for ERK activity by either ERK2 immune complex kinase assay (upper panel) or western blotting with phospho-ERK1/2 antibody (lower panels). Blots were stripped and reprobed with ERK1/2 antibody (lower panel). (B) The data from multiple ERK2 immune complex kinase assay experiments are shown as fold activation over basal (untreated cells) (n=3 +/- SEM); KCl alone (black bars), KCl plus H89 (hatched bars), KCl plus nifedipine (white bars). (C) PC12 cells were left untreated as control (cont) or treated with KCl (60 mM) or Forskolin (10 μM) (Forsk.) for 10 min in the presence or absence of the PKA inhibitor KT5720 (1 μM). Cells were harvested and immune complex kinase assays were performed. A representative autoradiogram is shown. (D) PC12 cells were treated with KCl (60mM) for ten minutes either in the absence (cont) or presence of W12 (50 μM) or W13 (50 μM). Western blots were performed using either phospho-ERK1/2 or ERK1/2 antibodies. (E) PC12 cells were treated with ionomycin (5 μM), with or without H89 pretreatment (10 μM), for five minutes as indicated, and western blots performed using phospho-ERK1/2 and ERK1/2 antibodies.

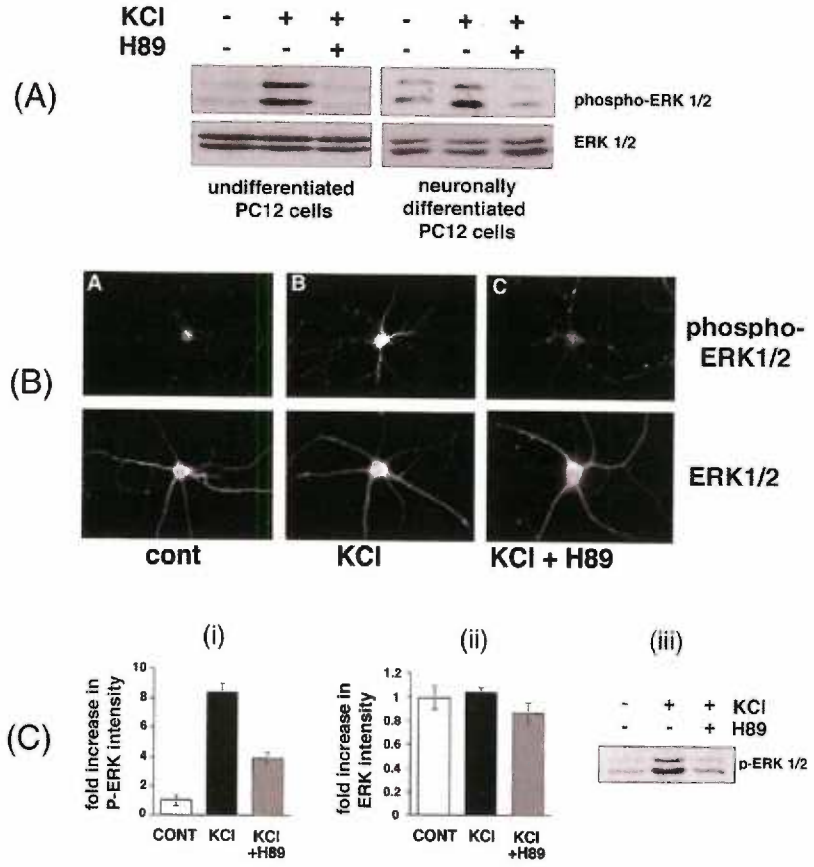

Figure 3.3. **Depolarization of neuronally-differentiated PC12 cells and hippocampal neurons activates ERKs via PKA.** (A) undifferentiated PC12 cells (left panel) and neuronally-differentiated PC12 cells (right panel) were treated with KCl (60mM) for 10 min in the absence or presence of the PKA inhibitor, H89 (10μM) as indicated. Cells were then lysed and equal protein amounts per treatment condition used for western blot with phospho-ERK1/2 or ERK1/2 antibody. (B) Immunofluorescence of hippocampal neurons with phospho-ERK1/2 antibody (upper panel) or ERK1/2 antibody (lower panel). Cells were untreated (cont), or treated for ten minutes with KCl (60mM) in the absence (KCl) or presence (KCl + H89) of the PKA inhibitor H89. Panels indicates representative images of cells treated in parallel. (C) (i) Quantitation of data represented in (B, upper panel). Bars indicate intensity of fluorescence in the cell bodies of phospho-ERK1/2 stained neurons expressed as fold increase over untreated cells (mean +/- SEM n>25 cells per condition). (ii) Quantitation of data represented in (B, lower panel). Bars indicate intensity of fluorescence in the cell bodies of ERK1/2 stained neurons expressed as fold increase over untreated cells (mean +/- SEM n>25 cells per condition). (iii) Western blot of hippocampal neurons. Cells were treated as indicated for ten minutes in the absence or presence of H89. Cells were then lysed and equal protein amounts per treatment condition used for western blot with phospho-ERK1/2 antibody.

data suggest that the PKA-dependent activation of ERKs may be a common target for calcium influx signaling in neuronal systems.

Neuronal depolarization activates B-Raf, but not Raf-1, via PKA. Previous studies have identified Raf-1 as the primary Raf isoform involved in signaling to ERKs, particularly with respect to growth factor actions in non-neuronal cells. In contrast, B-Raf is the major neuronal Raf isoform, being highly expressed within the CNS and PC12 cells. The involvement of these Raf isoforms in neuronal calcium-mediated signaling has not been well established. We therefore examined the contribution of these two isoforms to depolarization-induced calcium signaling. Membrane depolarization of PC12 cells activated B-Raf, an effect that was completely blocked by pre-treatment with the PKA inhibitor, H89 (Figure 3.4A). In contrast, Raf-1 was not activated by depolarization, even in cells where depolarization induced a robust phosphorylation of ERKs (Figure 3.4B).

Neuronal depolarization activates Rap1 via PKA, and induces formation of a Rap1/B-Raf signaling complex. B-Raf can be activated by both Ras and Rap1. This mechanism by which this occurs involves the recruitment of B-Raf to the membrane by either active Rap1 or Ras where it can then subsequently be stimulated. A requirement for PKA in the activation of ERKs suggests a possible role for Rap1. Thus, we next determined the relative contribution of Rap- and Ras-dependent pathways by first examining a requisite step in B-Raf activation: association of the kinase with its upstream small G-protein activator. Membrane depolarization of PC12 cells led to a strong association of Rap1 with B-Raf (Figure 3.5A.i). This effect was similar to that seen with RapV12, a constitutively activated mutant of Rap1. Furthermore, the B-Raf that associated with Rap1 was capable of phosphorylating MEK-1 (Figure 3.5A.ii). In contrast, while NGF led to a robust association of Ras with B-Raf, membrane depolarization had no effect (Figure 3.5B.i).

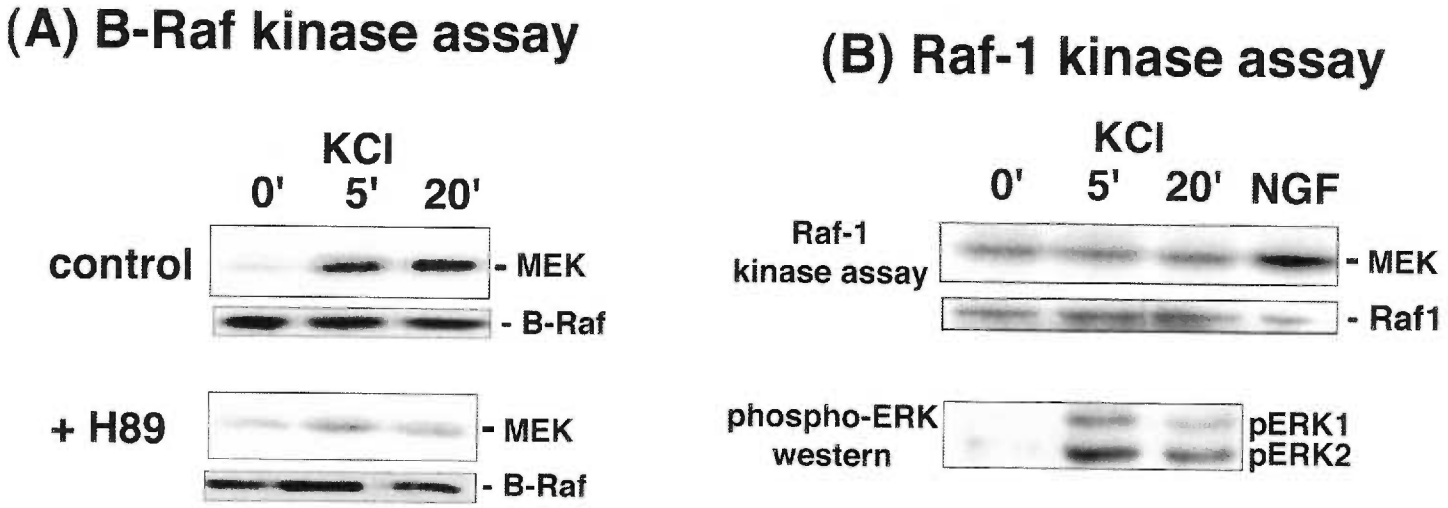

Figure 3.4. **Depolarization activates B-Raf, but not Raf-1, via PKA in PC12 cells.** (A) PC12 cells were treated with KCl (60 mM, 5 and 20 min), or left untreated (0 min), in the absence or presence of the PKA inhibitor, H89 as indicated. B-Raf was immunoprecipitated from equal amounts of lysate per condition and immune-complex assays performed using MEK-1 as a substrate. A representative autoradiogram with the position of MEK-1 is shown. Bottom panels indicate equal protein amounts of B-Raf per treatment as assayed by western blot. (B) PC12 cells were treated as in (A), and Raf-1 immune complex kinase assays performed following immunoprecipitation of Raf-1. Bottom panels indicate equal protein amounts of Raf-1 per treatment as assayed by western blot. NGF treated cells (NGF, 50 ng/ml) served as a positive control. The lower panel shows the phosphorylation of ERKs in the same whole cell lysates, using phospho-specific ERK1/2 antibody.

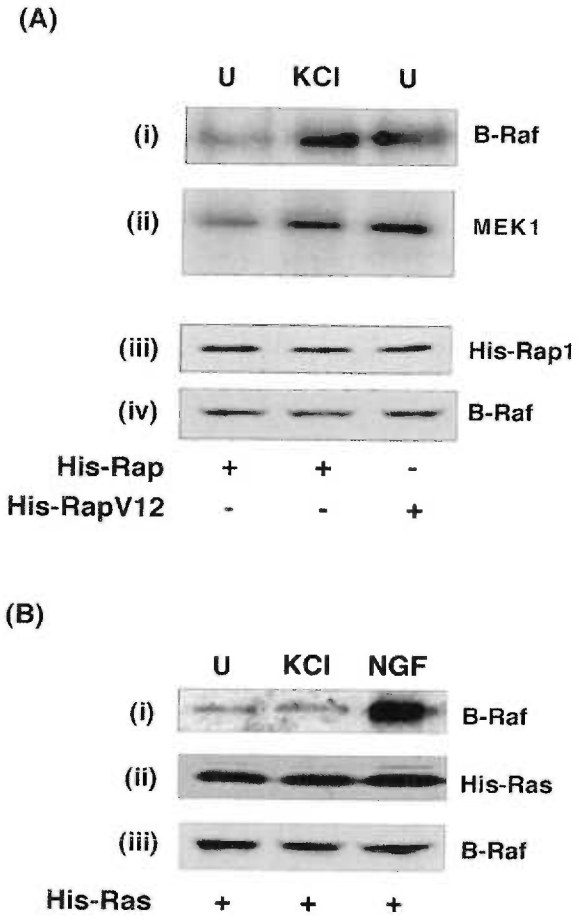

Figure 3.5. **Depolarization leads to association of Rap1, but not Ras, with B-Raf in PC12 cells.** (A) PC12 cells were transfected with polyhistidine-tagged Rap1b (His-Rap) and either left untreated (U) or treated with KCl (60 mM, 10 min), or transfected with a constitutively activated mutant (His-RapV12) as indicated. Cells were lysed and Ni-NTA Agarose beads were used to precipitate His-tagged proteins. (i) Endogenous B-Raf protein associating with the His-Rap1 protein was detected within the precipitated complex by Western blot. The position of B-Raf protein is indicated. (ii) The level of B-Raf activity within the precipitated complex was assayed by immune complex assay using MEK as an *in vitro* substrate. The level of MEK phosphorylation is shown. Controls showing the level of His-Rap within the complex precipitated by Ni-NTA Agarose, (iii), and the levels of endogenous B-Raf within whole cell lysates, (iv), are indicated. (B) PC12 cells were transfected with polyhistidine-tagged Ras (His-Ras) and treated with KCl (60 mM) for ten minutes or left untreated (U). NGF-treated cells (10 min) served as a positive control (NGF). Cells were lysed and Ni-NTA Agarose beads were used to precipitate His-tagged proteins. (i) Endogenous B-Raf protein associating with the His-Ras was detected within the precipitated complex by Western blot. Controls showing the level of His-Ras within the complex precipitated by Ni-NTA Agarose, (ii), and the levels of endogenous B-Raf within whole cell lysates, (iii), are indicated.

The data above suggests that calcium influx may target a Rap1-dependent signaling pathway. Therefore, we directly examined the activation of Rap1 by monitoring GTP-loading. Depolarization of PC12 cells led to a marked increase in Rap1 GTP loading (Figure 3.6A). This effect was similar in magnitude to that observed with both the activator of adenylate cyclase, forskolin, and the constitutively active form of Rap1, RapV12 (Figure 3.6A). Moreover, the depolarization-induced stimulation of Rap1 was completely reversed by pretreatment with the PKA inhibitor, H89 (Figure 3.6B).

Discussion

The present study describes a novel mechanism by which calcium influx may stimulate ERKs in neurons (Figure 3.7). Depolarization of PC12 cells, which stimulates calcium influx via L-type calcium channels induced the binding and activation of B-Raf by Rap1. Moreover, the stimulation of both Rap1 and B-Raf occurred via PKA. The major downstream consequence of Rap1/B-Raf signaling is the stimulation of ERKs (Vossler, et al., 1997). We further demonstrated that activation of ERKs following depolarization-mediated calcium influx in both PC12 cells and hippocampal neurons occured via a PKA-dependent pathway. This is consistent with the involvement of a Rap1/B-Raf signaling cascade. While previous studies have either shown or inferred a role for Ras/Raf1 signaling to ERKs in neurons, the present data identify the Rap1/B-Raf pathway as an important additional target for neuronal calcium signaling. Both proteins are highly expressed in the CNS, possibly at post-synaptic sites. Additionally, B-Raf expression is specifically up-regulated during LTP (Thomas, et al., 1994). As such, the Rap1/B-Raf pathway may be an important regulator of activity-dependent neuronal function.

Although a requirement for Ras in calcium-induced ERK activation has been previously reported (Egea, et al., 1999; Rosen, et al., 1994; Rusanescu, et al., 1995), we were not

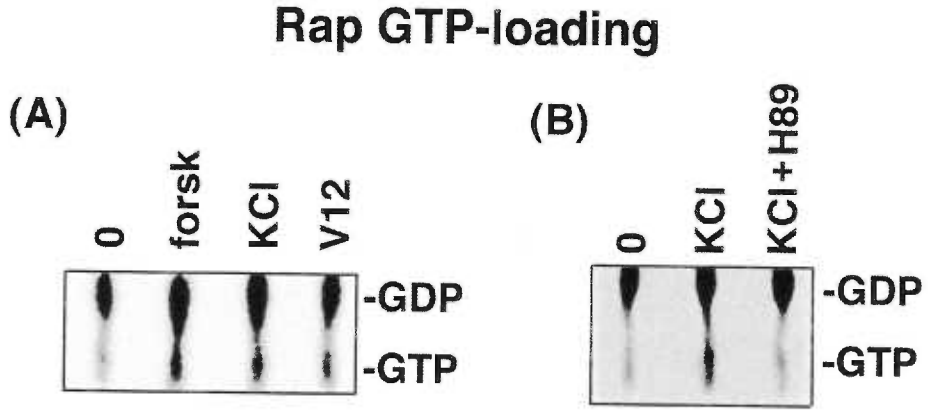

Figure 3.6. **Depolarization activates Rap1 via PKA in PC12 cells.** a, PC12 cells were transfected with Flag-tagged Rap1b or Flag-tagged RapV12. Rap GTP-loading assays were performed as described. Cells were treated with either KCl (60mM) or forskolin (10 μM) for 10 min as indicated. A representative autoradiogram is shown with GDP and GTP markers indicated. b, A Rap GTP-loading assay was performed as in a. Cells were either untreated (0) or treated with KCl (60mM) for 10 min in the absence (KCl) or presence (KCl+H89) of the PKA inhibitor, H89.

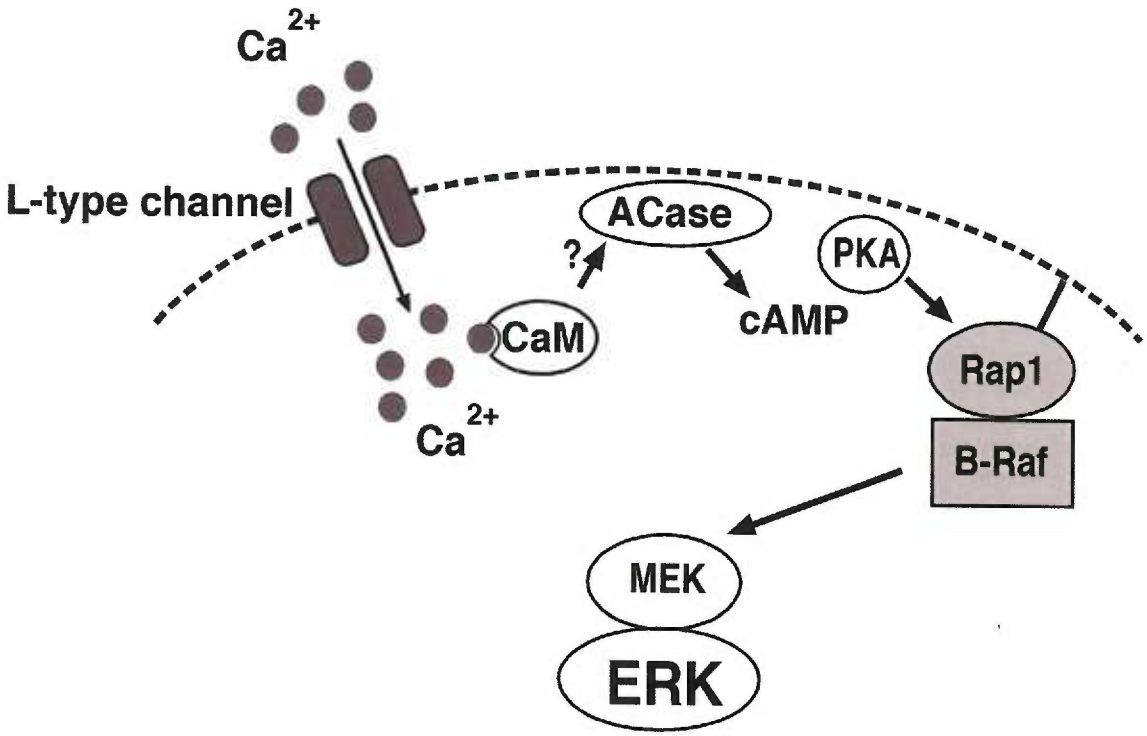

Figure 3.7. **Model of depolarization-mediated ERK signaling via PKA and Rap1/B-Raf.** Membrane depolarization of PC12 cells induces opening of L-type calcium channels leading to calcium influx. Calcium can then activate a membrane Rap1/B-Raf signaling pathway to cytoplasmic ERKs via PKA. This requirement for PKA may reflect the regulation of cAMP signaling via stimulation of calmodulin-activated adenylate cyclases at the membrane. CaM, calmodulin; ACase, adenylate cyclase.

able to show functional coupling of Ras to B-Raf, nor was Raf-1 activated. Such differences may reflect variability between the properties of PC12 cell clones used (e.g. stably-transfected versus wild-type PC12 cells). It is also conceivable that while depolarization may activate Ras, the subsequent actions of Ras may be Raf-independent. Indeed, in a recent report, while Ras was shown to be required for ERK activation by depolarization, no increase in activity of any Raf isoform (A-Raf, B-Raf, c-Raf-1) was observed (Egea, et al., 1999). One possibility is that the downstream actions of Ras on any of the Raf isoforms may be blocked. A potential inhibitor of Ras is Rap1 itself (Bos, 1998; Cook, et al., 1993). Rap1 was first identified and perhaps is best characterized as an inhibitor of Ras-dependent signaling (Kitayama, et al., 1989). Furthermore, Rap1 activation by PKA can account for the ability of cAMP to antagonize Ras signaling (Vossler, et al., 1997). Therefore, it is possible that depolarization-induced activation of Rap1 may activate Ras-independent pathways to ERK while inhibiting Ras-dependent pathways.

The signaling pathways stimulated by neuronal calcium are probably determined by a number of factors that regulate calcium influx. For example, the route of calcium influx may be important (Gallin and Greenberg, 1995; Ginty, 1997). Indeed, studies in hippocampal cells have demonstrated that calcium influx via either L-type or NMDA channels may have qualitatively different actions on gene expression (Bading, et al., 1993; Hardingham, et al., 1999). In the context of ERK activation, the route of calcium entry may also be an important factor in determining signaling events. For example in an early study, Ras-dependent pathways were identified following ionophore-induced calcium influx (Rosen, et al., 1994). In the present study, we show data that suggest different actions of ionophore versus L-type channel calcium influx. Thus, ionomycin stimulated ERKs via a PKA-independent pathway, consistent with a role for Ras, while depolarization activated a PKA-dependent Rap1/B-Raf signaling pathway.

In the present study we have used three different systems (undifferentiated PC12 cells, neuronal PC12 cells and hippocampal neurons) to examine depolarization-mediated activation of ERKs. While stimulation of a PKA-dependent pathway to ERKs appears to be a conserved mechanism common to all three cell types, it is possible that differences in calcium flux through L-type channels may differentially control ERK activation. Indeed, in hippocampal cells, depolarization appears to activate equally both PKA-dependent and -independent pathways to ERKs. This may reflect differences in the magnitude of calcium influx, with perhaps large increases in calcium entry via L-type channels leading to activation of both Ras and PKA/Rap1 pathways. Alternatively, the submembrane localization of downstream signaling molecules relative to the source of calcium influx may be an important factor that determines coupling to intracellular signaling cascades (Deisseroth, et al., 1996). For example in PC12 cells, calcium influx via L-type channels may be very efficiently coupled to PKA-dependent pathways, while in hippocampal cells calcium influx may be able to activate both PKA-dependent and -independent pathways equally. Such a model would be consistent with the emerging theme that compartmentalization of signaling modules can dictate cellular events, particularly with respect to PKA signaling (Colledge and Scott, 1999; Pawson and Scott, 1997)

Ultimately, it is likely that both Ras and Rap1 are important targets for neuronal calcium actions and the relative contribution of either pathway may be determined by cell-type- and stimulus-specific factors. Indeed the ability of neuronal depolarization to activate both pathways may have important implications in regulating the specificity of calcium-mediated signaling and gene expression, as has been described for NGF (York, et al., 1998). Moreover, the ability to activate both Ras- and PKA/Rap1-dependent signaling pathways may allow neurons to translate specific electrical impulses into distinct intracellular signaling events (Fields, et al., 1997). This may be important in regulating the temporal

profile of ERK signaling and subsequent transcriptional events (Buonanno and Fields, 1999).

Given our findings that Rap1 may be a target for neuronal calcium, it is important to consider the mechanisms by which it may be activated. In previous studies, we have shown that both cAMP and NGF stimulate Rap1 in a PKA-dependent manner (Yao, et al., 1998; York, et al., 1998). These findings, together with our present data showing that the activation of both Rap1 and its downstream effector, B-Raf are reversed by inhibition of PKA, argue for a role for PKA-regulated Rap1 activation. Our work with NGF suggests that the Rap1 GEF, C3G, may be important in this context (York, et al., 1998). However, recent studies have identified other novel Rap1-GEFs that are activated independently of PKA. Interestingly these appear to be stimulated directly by both calcium and cAMP binding (de Rooij, et al., 1998; Kawasaki, et al., 1998; Kawasaki, et al., 1998). Moreover, they are highly expressed in localized regions within the CNS. A role for such factors in neuronal calcium regulated Rap1 activation is therefore entirely possible. Clearly, further studies examining the role of Rap1 activation and, in particular the relative contribution of specific Rap1-GEFs, in neurons are warranted.

A requirement for PKA in mediating the actions of depolarization on Rap1/B-Raf activity suggests a role for calmodulin-stimulated adenylate cyclases (Xia and Storm, 1997). Consistent with this, in the present study we have demonstrated a requirement for calmodulin in the actions of depolarization. Calmodulin-stimulated adenylate cyclases are specifically expressed within the CNS and can be activated by depolarization and neuronal activity. In both neurons and PC12 cells calcium influx has been shown to increase cAMP levels and stimulate PKA activity (Agnihotri, et al., 1997; Xia and Storm, 1997). As such many activity dependent neuronal events such as regulation of synaptic plasticity and gene expression appear to require PKA activity (Abel, et al., 1997; Ginty, et al., 1991; Huang

and Kandel, 1998; Impey, et al., 1996; Impey, et al., 1999; Nguyen and Kandel, 1996; Xia, et al., 1996). Moreover, a number of recent reports also describe a role for ERKs in these processes (Atkins, et al., 1998; Coogan, et al., 1999; English and Sweatt, 1997; Impey, et al., 1998; Kawasaki, et al., 1999). Given our present findings, it is possible that some of these actions may reflect the involvement of PKA-dependent Rap1/B-Raf signaling to ERKs. For example, studies examining the mechanisms of long-term facilitation in *Aplysia* have also indicated that regulation of ERK activity by PKA may be an important component of this process (Martin, et al., 1997). Such a model would also be consistent with a role for both Rap1 and B-Raf. Thus, the identification of a linear PKA-ERK signaling pathway downstream of neuronal calcium may have important implications for our understanding of the regulation of neuronal function.

In a recent study, it was demonstrated that PKA was specifically required for the nuclear translocation of ERKs in neuronal cells via an, as yet, undefined mechanism (Impey, et al., 1998). It has been widely established that cytoplasmic stimulation of ERKs is necessary for their subsequent cellular relocalization (Khokhlatchev, et al., 1998; Marshall, 1995). Hence, our findings suggest that PKA-dependent activation of ERKs themselves may contribute to their nuclear translocation (Yao, et al., 1998). However, based on this, we cannot address nor rule out an additional role for PKA in ERK translocation. Nevertheless, our results together with the earlier described study (Impey, et al., 1998) do point to important multiple roles for PKA in the control of neuronal ERK function.

In conclusion, we have identified a PKA-dependent activation of a Rap1/B-Raf signaling pathway downstream of neuronal depolarization. This pathway may be important in neuronal calcium signaling to ERKs. As such targeting of a PKA/Rap1/B-Raf pathway may regulate a number of activity dependent events such as changes in gene expression and synaptic plasticity. Furthermore, since activation of Rap1/B-Raf via PKA is a pathway

shared by neurotrophins, cAMP and depolarization (Vossler, et al., 1997; Yao, et al., 1998; York, et al., 1998), we suggest that this pathway may underlie the synergy between these factors in regulating neuronal function.

CHAPTER FOUR

Calcium and Cyclic AMP signals differentially regulate CREB function via a Rap1-ERK pathway

SAVRAJ S. GREWAL[1,2], DANIEL M. FASS[1], CINDY L. ELLIG[1],
HONG YAO[1], RICHARD H. GOODMAN[1], PHILIP J.S. STORK[1,2]

[1]Vollum Institute, L474, and [2]Department of Cell and Developmental Biology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201.

Published in *The Journal of Biological Chemistry* (2000) **275**, 34433-34441

Summary

Two major intracellular signals that regulate neuronal function are calcium and cyclic AMP (cAMP). In many cases, the actions of these two second messengers involve long-term changes in gene expression. One well studied target of both calcium and cAMP signaling is the transcription factor CREB. Multiple signaling pathways have been shown to contribute to the regulation of CREB-dependent transcription, including both protein kinase A (PKA)- and mitogen activated protein/extracellular signal-regulated kinase (MAP kinase or ERK)-dependent kinase cascades. We have previously described a mechanism by which cAMP and calcium influx may stimulate ERKs in neuronal cells. This pathway involves the PKA-dependent activation of the Ras-related small G-protein, Rap1, and subsequent stimulation of the neuronal Raf isoform, B-Raf. In this study, we examined the contribution of the Rap1-ERK pathway to the control of gene transcription by calcium influx and cAMP. Using the PC12 cell model system, we found that calcium influx and cAMP both stimulated CREB-dependent transcription via a Rap1-ERK pathway, but this regulation occurred through distinct mechanisms. Calcium-mediated phosphorylation of CREB through the PKA-Rap1-ERK pathway. In contrast, cAMP phosphorylated CREB via PKA directly but required a Rap1-ERK pathway to activate a component downstream of CREB phosphorylation and CBP recruitment. These data suggest that the Rap1/B-Raf signaling pathway may have an important role in the regulation of CREB-dependent gene expression in neurons.

Introduction

Two major intracellular signals that regulate neuronal function are calcium and cyclic adenosine monophosphate (cAMP). For example, both activation of G-protein coupled receptors linked to adenylate cyclase and stimulation of calcium influx via either receptor- or voltage-operated calcium channels can exert rapid actions on neurotransmission and neuronal excitability (O'Connor, et al., 1994; Wickman and Clapham, 1995). However, in many cases, the actions of these two second messengers involve slower, long-term changes in gene expression (Ghosh and Greenberg, 1995; Montminy, 1997). These effects are often mediated by stimulation of intracellular signal transduction pathways that regulate the activity of multiple transcription factors. One well studied target of both calcium and cAMP is the transcription factor cAMP-responsive element binding protein (CREB) (Shaywitz and Greenberg, 1999). Regulation of this protein may be important in mediating changes in synaptic plasticity and neuronal survival (Bonni, et al., 1999; Martin and Kandel, 1996; Riccio, et al., 1999).

CREB binds as a dimer to a conserved cyclic-AMP response element (CRE) found in the promoters of numerous eukaryotic genes (Montminy, 1997). Phosphorylation of serine-133 is a critical event in CREB activation (Yamamoto, et al., 1988), which induces an increase in CREB transactivation potential by allowing the recruitment and binding to co-activators such as CREB-binding protein (CBP) (Chrivia, et al., 1993; Kwok, et al., 1994). Early studies identified PKA as a major physiological kinase responsible for Ser-133 phosphorylation (Gonzalez and Montminy, 1989). Consequently, one prevailing model of CREB regulation is that, following increases in intracellular cAMP levels and activation of PKA, the catalytic subunit of PKA translocates into the nucleus and phosphorylates CREB, leading to the stimulation of gene transcription (Hagiwara, et al.,

1993). Subsequent studies have identified additional CREB kinases, including members of the calcium/calmodulin-dependent kinase (CaMK) family (Dash, et al., 1991; Deisseroth, et al., 1996; Matthews, et al., 1994; Sheng, et al., 1991; Sun, et al., 1994) and the extracellular signal-regulated kinase (ERK)-stimulated RSK and MSK kinases (Deak, et al., 1998; Impey, et al., 1998; Xing, et al., 1996). These kinase families have been reported to mediate the actions of both calcium and growth factors on CREB phosphorylation. However, it is becoming increasingly clear that signaling events in addition to CREB phosphorylation are required for full CREB-dependent transcription (Shaywitz and Greenberg, 1999). For example, processes such as recruitment and regulation of co-activators and coupling to the basal transcription machinery may present additional targets for kinase action. Indeed, the activity of the transcriptional co-activator CBP has been reported to be regulated by a variety of kinases including PKA, CaMKIV and ERK, possibly by direct phosphorylation (Ait-Si-Ali, et al., 1999; Chawla, et al., 1998; Hardingham, et al., 1999; Hu, et al., 1999; Kwok, et al., 1994; Liu, et al., 1998; Liu, et al., 1999; Xu, et al., 1998). As such, it is likely that the multiple actions of different signaling pathways may ultimately contribute to the stimulation of full CREB-dependent transcription by both calcium and cAMP.

We have previously described a novel mechanism by which cAMP and calcium influx may stimulate ERKs in neuronal cells (Grewal, et al., 2000; Vossler, et al., 1997). This pathway involves the PKA-dependent activation of the Ras-related small G-protein, Rap1, and subsequent stimulation of the neuronal Raf isoform, B-Raf. Activation of this pathway results in robust stimulation of ERKs and ERK-dependent gene expression (Grewal, et al., 2000; Vossler, et al., 1997; York, et al., 1998). Both Rap1 and, in particular, B-Raf are highly expressed within the nervous system. Hence, stimulation of a Rap1-ERK pathway may contribute to neuronal cAMP and calcium signaling (Dugan, et al., 1999; Grewal, et al., 1999; Vossler, et al., 1997). Moreover, this pathway may allow for cross talk between

PKA and ERK signaling systems. The downstream consequences of stimulation of the Rap1-ERK pathway by calcium and cAMP are not fully understood. However, given the ability of both PKA and ERK to regulate CREB activity, it is possible some of the observed actions of PKA occur via cross talk through the Rap1-ERK pathway.

In this study, we examined the contribution of the Rap1-ERK pathway to the control of gene transcription by calcium influx and cAMP. Using the PC12 cell model system, we found that both stimulation of calcium influx and elevation of intracellular cAMP levels stimulated CREB-dependent transcription via the PKA-dependent Rap1-ERK pathway. Interestingly, this appears to occur through distinct mechanisms. Calcium used a PKA-Rap1-ERK pathway to mediate phosphorylation of CREB. In marked contrast, cAMP phosphorylated CREB via PKA directly but required a Rap1-ERK pathway to mediate an event that was downstream from Ser-133 phosphorylation and CBP recruitment to achieve full transcription. These data suggest a revised model for cAMP regulation of CREB in which the co-ordinate action of both PKA-dependent phosphorylation of CREB and Rap1-ERK stimulation of a downstream target are required for full transcription.

Materials and methods

Materials. PC12-GR5 cells were kindly provided by R. Nishi, Oregon Health Sciences University, Portland, Oregon. Forskolin, 8-CPT-cAMP and PD98059 were purchased from Cal Biochem (Riverside, CA). Phosphorylation-specific and phosphorylation state-independent rat polyclonal CREB antibodies were purchased from New England Biolabs (Beverly, MA). Polyclonal antibody to B-Raf(C19) was purchased from Santa Cruz Biotechnology Inc.

Immunofluorescence assays. PC12 cells were treated as described and after the indicated times fixed in paraformaldeyde. Following permeablization in methanol and blocking in 5% normal goat serum (NGS), cells were incubated in primary antibody (1:2500 in 5%NGS) overnight at 4°C. Cells were incubated in secondary antibody (1:2500 Alexa 546 conjugated anti-rabbit) for one hour at room temperature. Cells were visualized using a Leica DMRB microscope. The intensity of CREB immunofluorescence was measured using NIH Image software. Briefly, signal intensity was quantitated as nuclear pixel density above background, and was expressed as fold increase over unstimulated cells.

Luciferase reporter gene assays. Cells were treated with the appropriate stimuli for four to five hours. Cells were then lysed and equal protein amounts of lysate per condition were assayed for luciferase as described. All experiments were performed with at least three independently treated plates per condition.

Results

Calcium and cAMP stimulate CRE-dependent transcription via a PKA-dependent Rap1-ERK pathway. We initially began examining the regulation of CREB function in PC12 cells using a CRE reporter system consisting of five reiterated CREs controlling the expression of luciferase (Chen, et al., 1995). Following transfection of the reporter into cells, we found that KCl depolarization-mediated opening of L-type calcium channels induced a marked stimulation of CRE-dependent transcription (Figure 4.1). Similarly, elevation of intracellular cAMP levels by treatment with the adenylate cyclase activator, forskolin, also stimulated transcription (Figure 4.1). In both cases, the increase in CRE-dependent transcription was reversed by either co-transfection of the

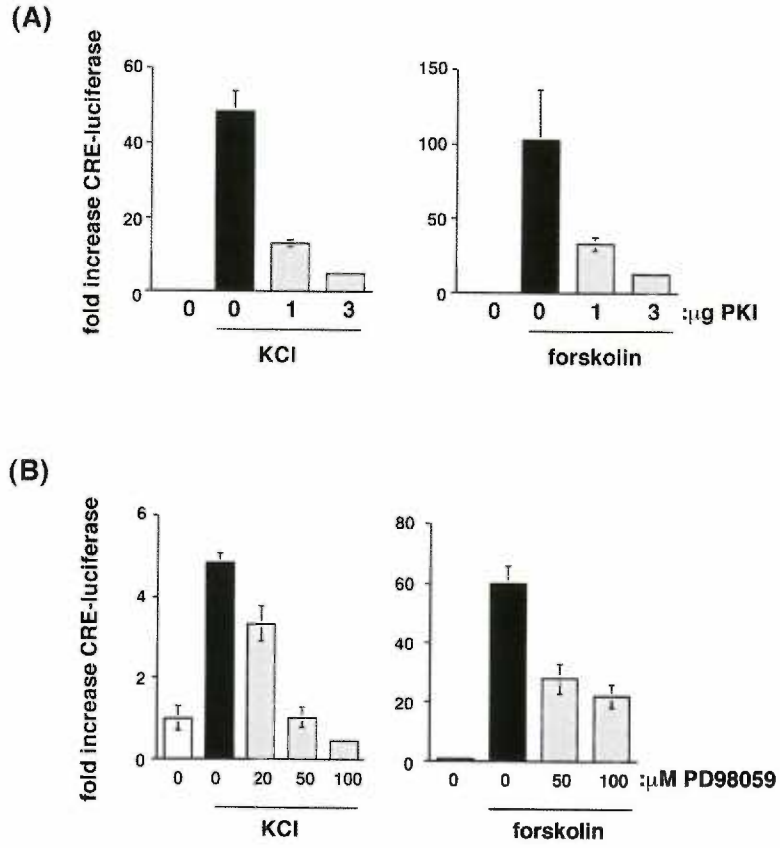

Figure 4.1. **Depolarization and forskolin stimulate CRE reporter via a PKA- and ERK-dependent mechanism.** (A) PC12 cells were transfected with a CRE-luciferase reporter and either 0, 1, or 3 μg PKI as indicated. Cells were then treated with either KCl (60 mM) or forskolin (10μM) for four hours as indicated and then harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of at least three independent treatments. (B) PC12 cells were transfected with a CRE-luciferase reporter. Cells were pretreated with 0, 20, 50, or 100 μM PD98059 as indicated. Cells were stimulated with either KCl (60 mM) or forskolin (10μM) for four hours as indicated and then harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments.

selective PKA inhibitor, PKI (Figure 4.1A), or pre-treatment with the selective MEK inhibitor, PD98059 (Figure 4.1B). These data suggest that both calcium influx and cAMP require PKA and ERK signaling events to regulate CREB function. We have previously demonstrated that, in PC12 cells, calcium and cAMP activate a PKA-dependent Rap1/B-Raf signaling pathway to stimulate ERKs (Grewal, et al., 2000; Vossler, et al., 1997). Therefore, the requirement for both PKA and ERK in the regulation of CRE-dependent transcription may reflect activation of this pathway. We investigated this using a specific inhibitor of Rap1, Rap1GAP1 (Rubinfeld, et al., 1991). This protein stimulates the conversion of Rap1 from its GTP-bound, active, to GDP-bound, inactive state. Previous work has demonstrated that overexpression of Rap1GAP1 proteins inhibit Rap signaling in PC12 cells (Jordan, et al., 1999). In addition, using an Elk-gal reporter system to monitor ERK activation, we found that the activation of Elk-dependent transcription by both KCl and forskolin in PC12 cells was inhibited by co-transfection of Rap1GAP1 (Figure 4.2A). Using the CRE-luciferase reporter system, we also found that the increase in transcription mediated by both KCl and forskolin was inhibited by Rap1GAP1 (Figure 4.2B). These data suggest that both calcium influx and cAMP regulate CREB-dependent transcription via activation of a PKA-dependent Rap1-ERK signaling cascade.

Calcium influx stimulates CREB Ser-133 phosphorylation via a PKA-Rap1-ERK pathway. CREB transcriptional activity is stimulated by the phosphorylation of Ser133. Numerous studies have demonstrated that this can occur through either PKA- or ERK-dependent pathways. Phosphorylation of Ser-133 may therefore be a target for Rap1-ERK signaling in the regulation of CRE activity. We examined the phosphorylation of CREB by calcium and cAMP in PC12 cells using an antibody that recognizes phospho-Ser133 CREB (Figure 4.3). Both KCl and forskolin induced a rapid and sustained phosphorylation of CREB as assayed by western blot. In both cases this was reversed by pretreatment with the PKA inhibitor, H89 (Figure 4.3). In contrast, the MEK inhibitor

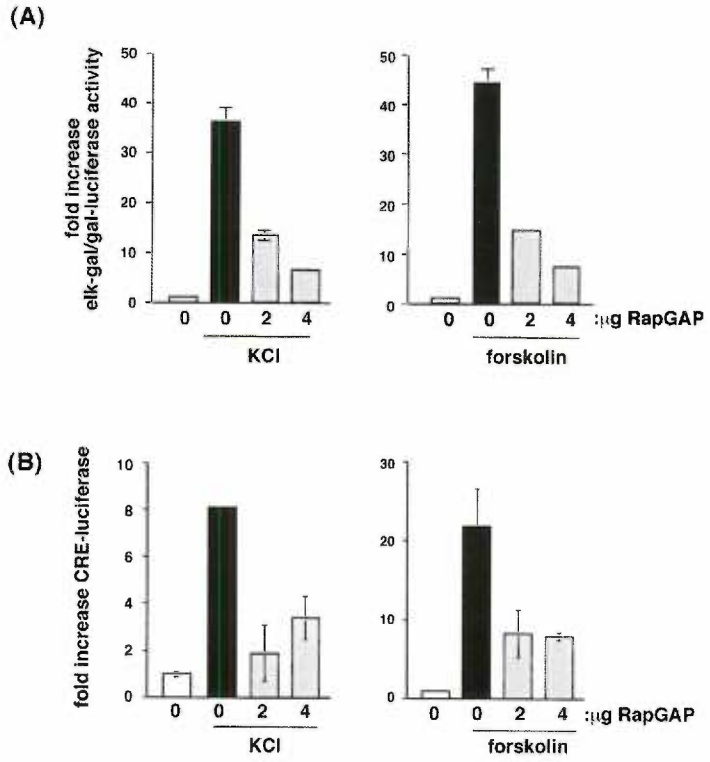

Figure 4.2. **Depolarization and forskolin stimulate both Elk-1- and CRE-dependent transcription via a Rap1-ERK pathway.** (A) PC12 cells were transfected with a gal-Elk1 plasmid and gal-luciferase reporter, and 0, 2 or 4 µg Rap1GAP1 as indicated. Cells were then treated with either KCl (60 mM) or forskolin (10µM) for four hours as indicated and then harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of at least three independent treatments. (B) PC12 cells were transfected with a CRE-luciferase reporter and either 0, 2, or 4 µg Rap1GAP1 as indicated. Cells were stimulated with either KCl (60 mM) or forskolin (10µM) for four hours as indicated and then harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments.

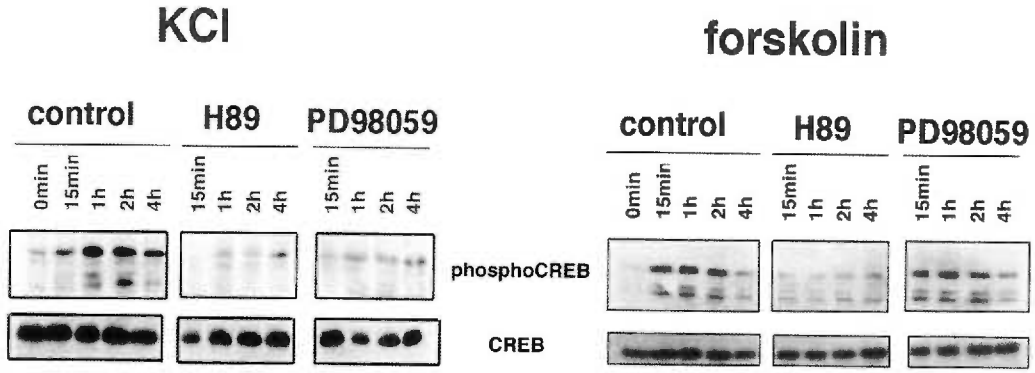

Figure 4.3. **Depolarization, but not forskolin, stimulates CREB Ser-133 phosphorylation via an ERK-dependent pathway.** PC12 cells were pretreated with either H89 (10μM) or PD98059 (50μM) as indicated. They were then stimulated with either KCl (60 mM) or forskolin (10μM) for 15min to 4 hours. At each timepoint, cell lysates were collected in SDS sample buffer, resolved by SDS-PAGE and analyzed for phospho-CREB by western blotting (upper panels). The blots were then stripped and reprobed for CREB (lower panels).

PD98059 blocked KCl-, but not forskolin-induced phosphorylation (Figure 4.3). We also examined CREB phosphorylation by immunofluoresence. Both KCl and forskolin induced an increase in phospho-CREB immunoreactivity (Figure 4.4). These effects were reversed by transfection of the selective PKA inhibitor, PKI (Figure 4.4B). Furthermore, as we observed with the western blots, PD98059 only blocked the increase CREB phosphorylation induced by KCl (Figure 4.4C). These data suggest that the requirement for Rap1-ERK action in CREB activation by calcium probably reflects an ERK-dependent phosphorylation of CREB. Activation of ERK signaling was not necessary for cAMP-mediated phosphorylation. In contrast, cAMP appeared to induce CREB phosphorylation via a direct PKA-dependent mechanism, consistent with the well described model of transcriptional regulation by cAMP.

Activation of a Rap1-ERK pathway is a conserved component of cAMP-dependent regulation of CREB transcription. The previous data indicate that cAMP uses a Rap1-ERK pathway to control CRE-dependent transcription, but that this regulation occurs at a site distinct from CREB Ser-133 phosphorylation. It is possible that this requirement for ERK activity is a function of the reporter system used. We therefore examined transcriptional regulation in a different promoter context, using a GAL4-CREB/ gal-luciferase reporter system. PC12 cells were co-transfected with a 5Xgal-luciferase reporter plasmid and a GAL4-CREB protein in which the CREB bZIP domain was replaced with an N-terminal GAL4 DNA binding and dimerization domain. Previous studies have demonstrated that this reporter system provides a measure of CREB-dependent transcription (Bonni, et al., 1995; Hagiwara, et al., 1992; Sheng, et al., 1991). Treatment of cells with the cAMP analogue, 8-CPT-cAMP or forskolin, or transfection of the PKA catalytic subunit (cPKA) all stimulated GAL4-CREB activity in PC12 cells (Figure 4.5). As observed with the CRE-luciferase reporter, these actions were reversed by the MEK inhibitor, PD98059 (Figure 4.5A, B). Inhibition of Rap1 activity by co-

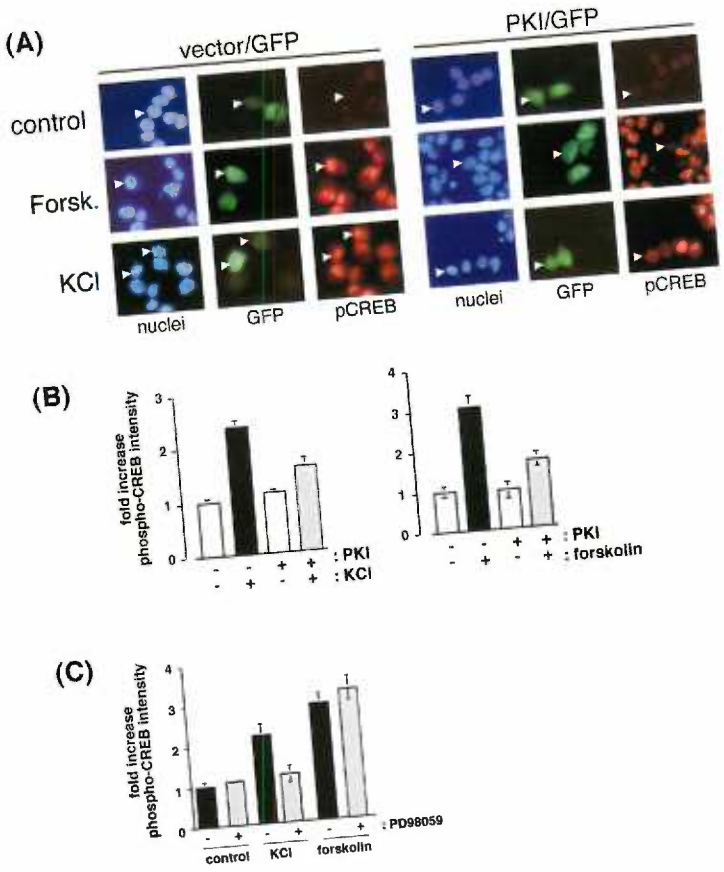

Figure 4.4. **Depolarization- and forskolin—dependent stimulation of phospho-CREB immunofluoresence.** (A) PC12 cells were transfected with either empty vector or PKI, and GFP as a marker, as indicated. Following stimulation with either KCl (60mM) or forskolin (10μM), cells were processed for phospho-CREB immunoreactivity as described. The arrowheads identify representative transfected cells. Left panels, Hoechst 33258 staining of nuclei; middle panel, GFP transfected cells; right panel, phospho-CREB immunofluorescence. (B) The intensity of phospho-CREB immunofluorescence in transfected cells was measured and expressed as fold increase over basal, unstimulated cells. Data are expressed as mean +/- SEM (n>50 cells per condition). (C) PC12 cells were pretreated with PD98059 (50μM) as indicated. Following stimulation with either KCl (60mM) or forskolin (10μM), cells were processed for phospho-CREB immunoreactivity as described. The intensity of phospho-CREB immunofluorescence in transfected cells was measured and expressed as fold increase over basal, unstimulated cells. Data are expressed as mean +/- SEM (n>50 cells per condition).

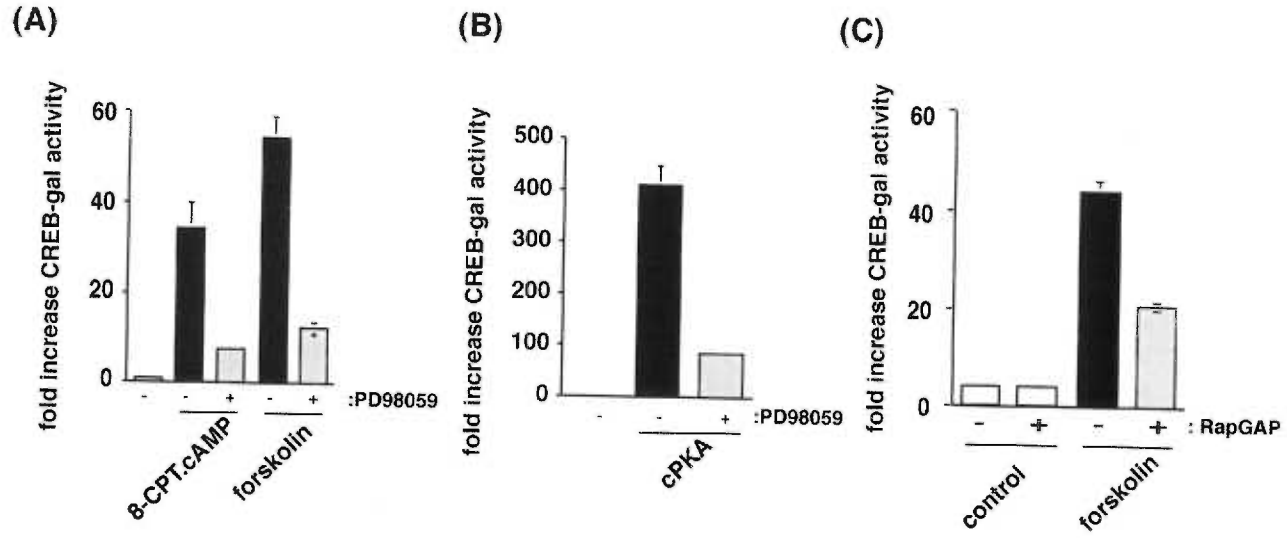

Figure 4.5. **Cyclic AMP stimulates CREB-dependent transcription via a Rap1-ERK pathway.** (A) PC12 cells were transfected with a GAL4-CREB plasmid and a gal-luciferase reporter. Cells were pretreated with PD98059 (50 μM) as indicated and then stimulated with either the cAMP analog, 8-CPT-cAMP (175 μM) or forskolin (10 μM) for four hours, before being harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments. (B) PC12 cells were transfected with a GAL4-CREB plasmid, a gal-luciferase reporter, and either empty vector or cPKA as indicated. Cells were incubated with PD98059 (50 μM) for four hours as indicated and then harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments. (C) PC12 cells were transfected with a GAL4-CREB plasmid, a gal-luciferase reporter, and either empty vector or Rap1GAP1 as indicated. Cells were left untreated or stimulated with forskolin (10 μM) for four hours as indicated and then harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments.

transfection of Rap1GAP1 also prevented forskolin-mediated stimulation of GAL4-CREB activity (Figure 4.5C).

We next examined whether the requirement for a Rap1-ERK pathway in cAMP signaling was specific to PC12 cells. We have previously demonstrated that the ability of cAMP to activate a Rap1-dependent signaling pathway to ERKs is dependent on the expression of B-Raf (Vossler, et al., 1997). Thus, we examined the regulation of CREB in two cell lines, Hek293 and NIH3T3, which differ in their expression of B-Raf. Hek293 cells express high levels of B-Raf. Elevation of cAMP by either direct stimulation of adenylate cyclase or activation of β-adrenergic receptors activates ERKs in these cells (data not shown). Using the CRE-luciferase reporter assay, we found that both forskolin and the β-adrenergic receptor agonist, isoproterenol, markedly stimulated CRE-dependent transcription (Figure 4.6A). Moreover, these effects were reversed by PD98059 (Figure 4.6A). In contrast, the phosphorylation of CREB Ser-133 induced by forskolin and isoproteronol was not blocked by PD98059 (Figure 4.6B). Thus, as in PC12 cells, cAMP stimulation of CRE-dependent transcription requires an ERK signaling component at a site distinct from CREB phosphorylation. We also investigated CRE-dependent transcription in the NIH3T3 cell line. PKA is unable to activate ERKs in these cells due to the absence of the Raf isoform, B-Raf (Vossler, et al., 1997). Transfection of B-Raf into these cells allows PKA to stimulate ERKs (Vossler, et al., 1997). In the absence of B-Raf, both forskolin and cPKA were able to activate CRE-dependent transcription (Figure 4.7). However, transfection of B-Raf potentiated the stimulatory actions of both forskolin and cPKA while having no effects on the basal levels of transcription (Figure 4.7A, B). These actions were independent of CREB phosphorylation: examination by western blot revealed that B-Raf had no effect on Ser-133 phosphorylation mediated by cPKA (Figure 4.7C). Together, these data suggest that the ability of PKA to activate a Rap1-ERK pathway is important for full CREB-dependent transcription by cAMP.

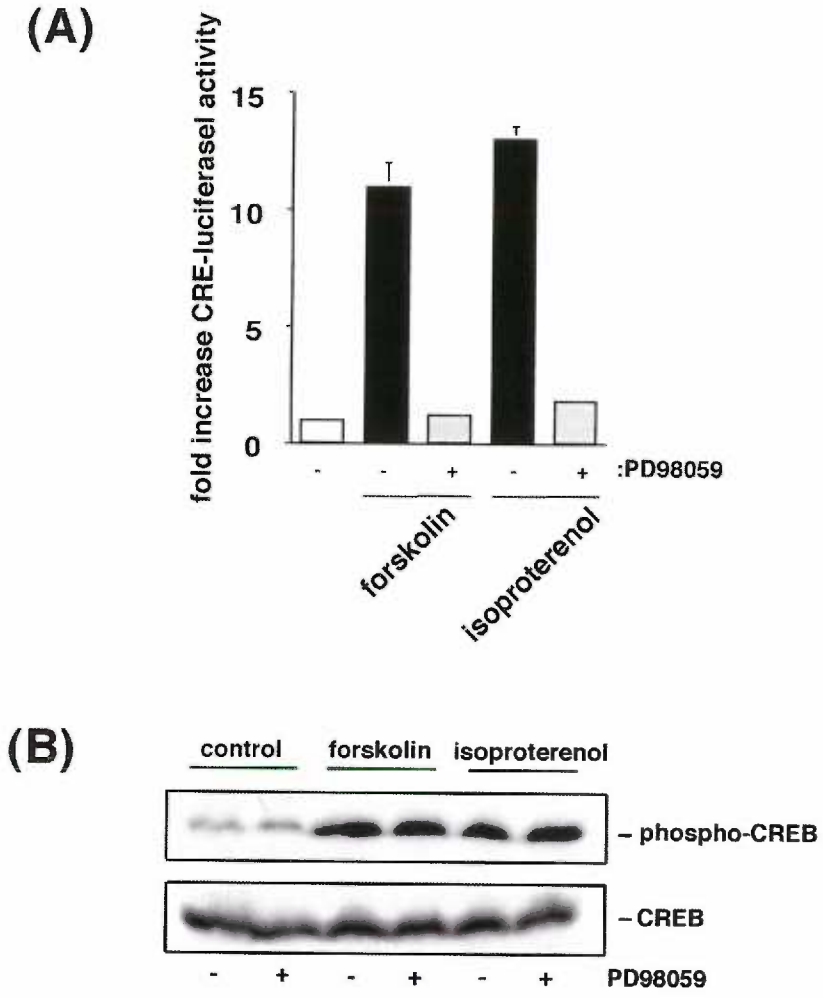

Figure 4.6. **Cyclic AMP regulates CREB activity via Rap1-ERK signaling in Hek293 cells.** (A) Hek293 cells were transfected with a 5XCRE-luciferase reporter. Cells were pretreated with PD98059 (50μM), as indicated, and then stimulated with either forskolin (10μM) or the β-adrenergic receptor agonist, isoproterenol (10μM), for four hours, before being harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments. (B) Hek293 cells were pretreated with PD98059 (50μM), as indicated, and then stimulated with either forskolin (10μM) or the β-adrenergic receptor agonist, isoproterenol (10μM) for 15min. Cell lysates were collected in SDS sample buffer, resolved by SDS-PAGE and either phospho-CREB (upper panel) or CREB (lower panel) western blotting performed.

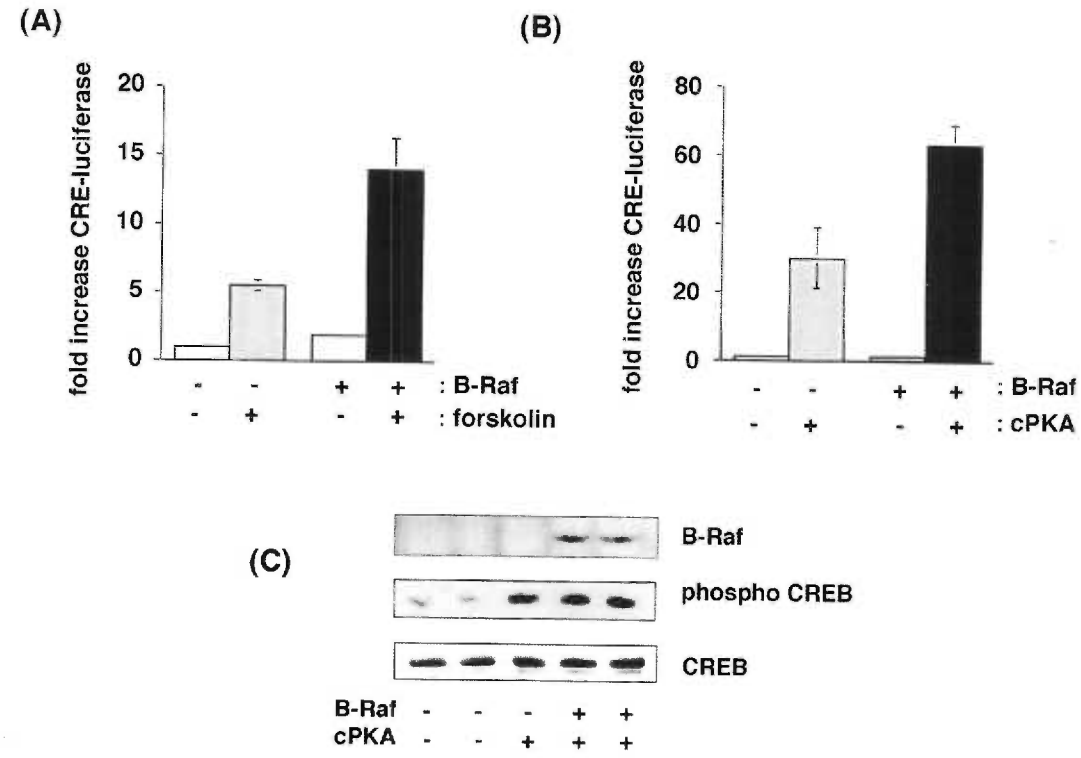

Figure 4.7. **Cyclic AMP regulates CREB activity via Rap1/B-Raf signaling in NIH3T3 cells.** (A) NIH3T3 cells were co-transfected with a 5XCRE-luciferase reporter and either empty vector or B-Raf as indicated. Cells were then left untreated or stimulated with forskolin (10 μM) for four hours as indicated, before being harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments. (B) NIH3T3 cells were co-transfected with a 5XCRE-luciferase reporter and combinations of empty vector, cPKA and B-Raf as indicated. Twenty-four hours later, cells were harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments. (C) NIH3T3 cells were co-transfected with combinations of empty vector, cPKA and B-Raf as indicated. Twenty-four hours later, cell lysates were collected in SDS sample buffer, resolved by SDS-PAGE and B-Raf (upper panel), phospho-CREB (middle panel) or CREB (lower panel) western blotting performed.

The Rap1-ERK pathway acts downstream from CREB Ser-133 phosphorylation and CBP recruitment to mediate full stimulation of CREB-dependent transcription by cAMP. The data described above suggest that a Rap1-ERK pathway contributes to the regulation of cAMP-mediated gene expression at a site downstream from CREB Ser-133 phosphorylation. One potential target for Rap1-ERK signaling is the control of co-activator function. For example, studies have demonstrated that CBP, a co-activator for CREB, can be regulated by a variety of kinase cascades including both PKA- and ERK-dependent signaling pathways. We examined this by using a recently described mutant of CREB, CREB-DIEDML. This mutant contains a substitution of six non-conserved amino acids (DIEDML) from the transcription factor sterol-responsive element binding protein (SREBP) that replace Ser-133 and the surrounding five amino acids (RRPSYR) in CREB (Cardinaux, et al., 2000). The resulting molecule allows constitutive, phosphorylation-independent binding to the co-activator CBP (Cardinaux, et al., 2000). Transfection of this CREB-DIEDML into F9 teratocarcinoma cells was previously shown to lead to constitutive stimulation of CRE-dependent transcription (Cardinaux, et al., 2000). We found that in PC12 cells, CREB-DIEDML also stimulated both CRE- and Fos-dependent transcription in an extracellular-signal-independent manner (Figure 4.8A). Fusion of the activation domain of CREB-DIEDML to the DNA binding domain of GAL4 allows it to induce constitutive stimulation of a gal-luciferase reporter in a signal-independent manner in PC12 cells ((Cardinaux, et al., 2000) and Figure 4.8B). Forskolin treatment potentiated GAL4-CREB-DIEDML activity further (Figure 4.8B). Interestingly, as observed with the GAL4-CREB protein, this stimulation by forskolin was reversed by PD98059 (Figure 4.8B). Calcium influx was unable to stimulate GAL4-CREB activity (data not shown). These findings are consistent with previous studies using GAL reporter systems, in which GAL4-CREB fusion proteins lacking the CREB bZIP

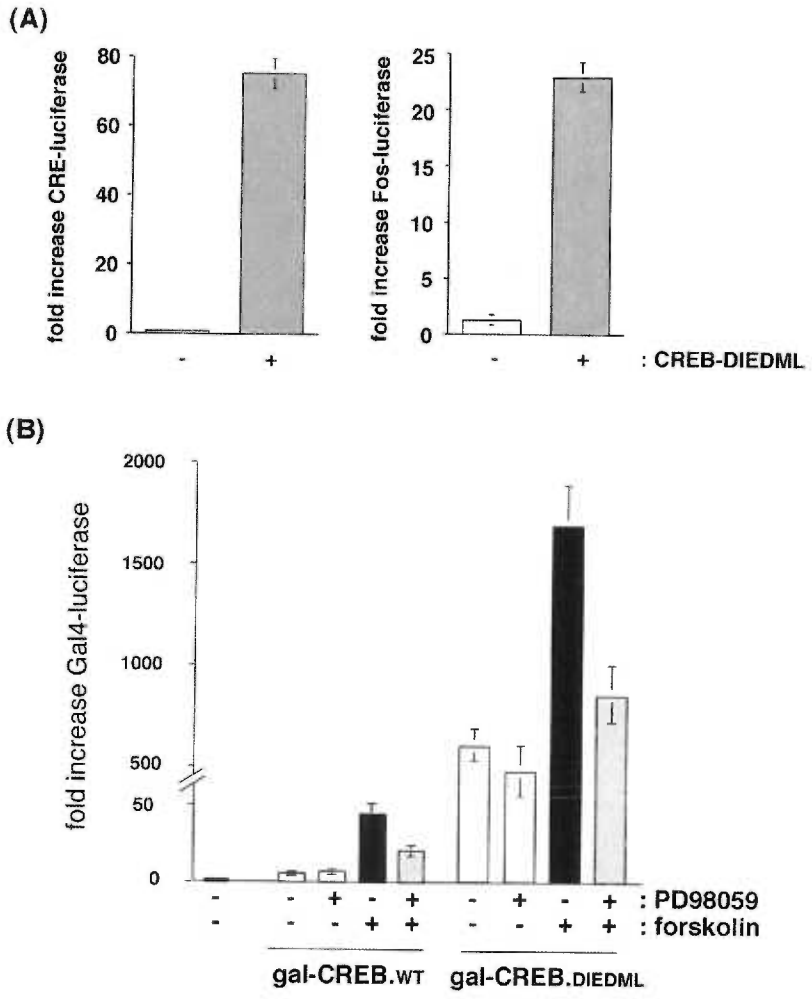

Figure 4.8. **Cyclic AMP regulation of CREB-DIEDML.** (A) PC12 cells were co-transfected with either vector or CREB-DIEDML as indicated and either a CRE-luciferase (left bar graph) or Fos-luciferase [Visvader, et al. 1988] (right bar graph) reporter. Twenty-four hours later, cells were harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells. Each bar represents the mean +/- SEM of three independent treatments. (B) PC12 cells were co-transfected with either GAL4-CREB or GAL4-CREB-DIEDML as indicated and a gal-luciferase reporter. Cells were pretreated with PD98059 (50μM) as indicated and then stimulated with forskolin (10μM) for four hours, before being harvested for luciferase assay. Data are expressed as fold increase over basal, unstimulated cells transfected with the gal-luciferase reporter only. Each bar represents the mean +/- SEM of three independent treatments.

domain are unresponsive to calcium influx (Bonni, et al., 1995; Sheng, et al., 1991). As a result, we were unable to evaluate the actions of calcium influx on CREB-DIEDML activity.

Discussion

In this study, we have demonstrated that activation of a Rap1-ERK signaling pathway in PC12 cells participates in the regulation of CREB-dependent transcription by both calcium influx and cAMP. However, this pathway seems to be used in distinct ways. Calcium-dependent phosphorylation of CREB requires Rap1-ERK signaling. In contrast, the cAMP-dependent stimulation of the Rap1-ERK pathway appears to contribute to transcription at step downstream of CBP recruitment (Figure 4.9).

Previous studies have demonstrated that a variety of kinase signaling pathways can differentially contribute to calcium influx-mediated phosphorylation of CREB in PC12 cells and neurons (Shaywitz and Greenberg, 1999). The exact mechanism used by calcium may depend on both cell-type and stimulus-specific factors. For example, in hippocampal cells, CaMKIV is reported to be a major calcium-regulated CREB kinase (Deisseroth, et al., 1996). In other cells, where CaMKIV is not expressed, ERK-dependent pathways may predominate. A recent study reported that calcium-mediated phosphorylation of CREB in PC12 cells occurred via the ERK–dependent activation of the CREB kinase, RSK-2 (Impey, et al., 1998). PKA was also required for regulating the nuclear translocation of ERK/RSK2, a prerequisite for CREB phosphorylation (Impey, et al., 1998). Our findings are consistent with a similar ERK-dependent mechanism of CREB phosphorylation following calcium influx. We also identify a requirement for PKA in this event. However, we suggest that this may reflect the ability of PKA to stimulate ERKs. While the Ras-ERK pathway is an important target for calcium signaling in many cell types, our previous

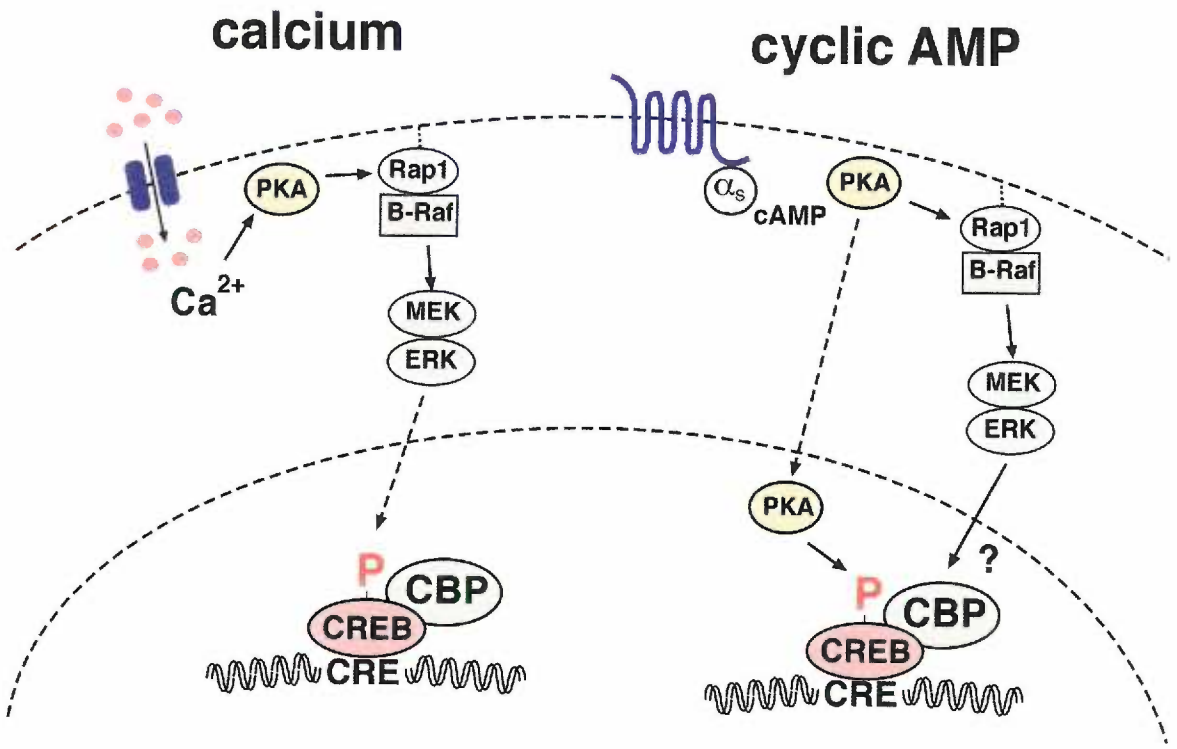

Figure 4.9. **A putative model of CREB regulation by calcium influx and cAMP in PC12 cells.** Calcium influx via L-type channels (left) stimulates CREB-dependent transcription via activation of a PKA-dependent Rap1-ERK pathway. The requirement for this pathway probably reflects a role for ERK signaling in calcium-induced CREB phosphorylation. Elevation of intracellular cAMP levels (right) also stimulates CREB function through the PKA-dependent Rap1-ERK pathway. In this case, cAMP phosphorylates CREB directly via PKA and utilizes the Rap1-ERK pathway to stimulate a component downstream of CBP recruitment. This latter step may reflect an action of ERK signaling on CBP function.

studies have indicated that PKA-dependent activation of a Rap1/B-Raf signaling pathway is the predominant mechanism by which calcium influx stimulates ERKs in the PC12 model system used in this report (Grewal, et al., 2000). A similar pathway may also be used in hippocampal cells (Grewal, et al., 2000; Martin, et al., 1997). Ultimately, PKA signaling may play multiple roles in the regulation of ERK–dependent pathways that lead to CREB phosphorylation following calcium influx.

A major finding of this study is that cAMP requires a Rap1-ERK signaling pathway to stimulate CREB-dependent transcription. Unlike the situation with calcium, this requirement for ERK activation appears not to involve its ability to mediate CREB Ser-133 phosphorylation. We find that stimulation of intracellular cAMP levels can lead to CREB phosphorylation via PKA, presumably through a direct action on Ser-133. Such an action has classically been thought to account for the ability of cAMP to stimulate CRE-dependent transcription (Montminy, 1997). Indeed, phosphorylation of CREB is often considered to be a measure of transcriptional activation by cAMP. Our data suggest that stimulation of a Rap1-ERK pathway may represent an additional required component of CREB regulation by cAMP. Importantly, this requirement for Rap1-ERK signaling was seen in multiple cell-types and in different promoter contexts. In addition, hormonal stimulation of β-adrenergic receptors coupled to adenylate cyclases used a similar mechanism to stimulate transcription. We therefore propose a revised model of cAMP regulation of CREB in which both PKA-mediated Ser-133 phosphorylation and stimulation of a PKA-Rap1-ERK pathway are required for full CREB-dependent transcription. Rap1 is highly expressed in neurons. Moreover, B-Raf expression is mainly restricted to neuronal and neuroendocrine cells. Therefore, this pathway is a potential regulator of neuronal CREB activity. Interestingly, previous reports have demonstrated that, in the context of calcium regulation of c-fos transcription in PC12 cells, a PKA-dependent signaling event distinct from CREB phosphorylation contributes to c-fos gene activation (Ginty, et al., 1991; Thompson, et al.,

1995). It is possible that these findings reflect a similar activation of Rap1-ERK signaling as described here.

An important consideration is the mechanism by which the Rap1-ERK pathway controls transcription downstream of CREB phosphorylation. One potential target is the transcriptional co-activator, CBP. CBP is a phosphoprotein that can potentially be regulated by a variety of kinase signaling pathways. However, it has been difficult to discriminate between mechanisms responsible for CREB phosphorylation versus CBP activation. One common approach has been to use gene-reporter assay systems in which GAL4-CBP fusion proteins target CBP to synthetic Gal promoters. Using these methods, studies have demonstrated that CBP-dependent transcriptional activity can be stimulated via a variety of signaling kinases including CaMKIV, PKA and ERKs (Ait-Si-Ali, et al., 1999; Chawla, et al., 1998; Hardingham, et al., 1999; Hu, et al., 1999; Kwok, et al., 1994; Liu, et al., 1998; Liu, et al., 1999; Xu, et al., 1998). However, it is unclear how well the GAL4-CBP fusion recapitulates the regulation of native CBP.

In this report, we have examined the actions of cAMP using a CREB mutant, CREB-DIEDML that contains a six amino acid substitution within its kinase inducible domain (Cardinaux, et al., 2000). Importantly, this mutant can bind CBP and stimulate CRE-dependent transcription in a phosphorylation-independent manner (Cardinaux, et al., 2000). We show that stimulation of cAMP/PKA signaling can further increase the transcriptional activity of this mutant. This increase appears to be indirect, involving PKA-dependent activation of the Rap1-ERK pathway. Given that CREB-DIEDML can constitutively bind to CBP and recruit it to the promoter, it is possible these effects involve modification of CBP activity itself. For example, some studies have demonstrated that the transcriptional activity of a GAL4-CBP fusion protein can be increased by ERKs (Janknecht and Nordheim, 1996; Liu, et al., 1998; Liu, et al., 1999). Furthermore, PKA

has been shown to augment GAL4-CBP function even when the sole consensus PKA phosphorylation site in CBP is mutated (Kwok, et al., 1994; Zanger, et al., 1999). These data are therefore also consistent with an indirect action of PKA, possibly via Rap1-ERK signaling. Intriguingly, in one study, the stimulatory actions of PKA on CBP were only observed in PC12 cells, a cell line that expresses B-Raf and in which cAMP can therefore stimulate a Rap1-ERK pathway (Swope, et al., 1996). In contrast, no effect of PKA was seen in COS-7 cells that express little or no B-Raf (Swope, et al., 1996).

Some reports have suggested that stimulation of Ras-ERK signaling cannot increase CBP activity (Chawla, et al., 1998; Hu, et al., 1999). In particular, Ras-dependent activation of RSK2 may actually antagonize CBP-dependent transcription (Nakajima, et al., 1996). These differences in the reported actions of ERK signaling may reflect contrasting roles for Ras- versus Rap1-dependent signaling. Thus, one model is that a Rap1-ERK pathway can increase CBP activity, whereas Ras signaling, possibly via RSK2, exerts an inhibitory effect. Given that a major action of PKA is to inhibit Ras signaling (Cook and McCormick, 1993; Wu, et al., 1993), cAMP modulation of CBP activity may reflect the net action of PKA-dependent inhibition of Ras and PKA-mediated activation of a Rap1-ERK pathway. CBP is also a co-activator for a variety of other transcription factors, e.g. Smads (Feng, et al., 1998; Janknecht, et al., 1998), STATs (Kurokawa, et al., 1998), nuclear hormone receptors (Kamei, et al., 1996). Therefore, regulation of its function through the Rap1-ERK signaling pathway may also provide a mechanism by which cAMP can control transcription independently of CREB.

An interesting finding of this study is that one signaling pathway (i.e. Rap1-ERK) can have distinct consequences on regulation of transcription depending on the type of signal (i.e. calcium influx versus cAMP). Moreover, stimulation of PKA activity is central to regulation of CREB by both calcium and cAMP, but through different downstream effects.

These particular effects may reflect stimulation of discrete spatial or subcellular pools of PKA (Colledge and Scott, 1999). For example, since Rap1 is membrane bound, PKA signaling at the membrane is probably required for activation of Rap1/B-Raf signaling and thus can regulate both calcium-induced phosphorylation of CREB and cAMP-mediated actions on CBP function through the Rap1-ERK pathway. In contrast, phosphorylation of CREB by cAMP probably involves a direct nuclear action of PKA. A similar scenario has been proposed for calcium signaling where distinct transcriptional responses are induced by cytoplasmic versus nuclear pools of calcium (Ginty, 1997; Hardingham, et al., 1997). These differential actions of PKA may be important in neurons where the intensity and spatial regulation of PKA activation may determine pathways used to stimulate CREB. For example discrete stimulation in dendrites may not be sufficient to lead to robust elevation of nuclear PKA, but could stimulate a Rap1-ERK pathway to phosphorylate CREB (Roberson, et al., 1999). In contrast, robust elevation of cAMP levels in the cell body could lead to direct nuclear PKA-mediated phosphorylation of CREB.

In conclusion, we find that stimulation of a PKA-dependent Rap1-ERK signaling pathway is important for the regulation of CREB function by both calcium and cAMP signals. Moreover, this mechanism of regulation may be important in controlling the neuronal action of CREB. Because CREB is a major regulator of processes such as synaptic plasticity and neuronal survival (Shaywitz and Greenberg, 1999; Walton and Dragunow, 2000), we propose that activation of Rap1-ERK signaling may be important in mediating these neuronal events.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

Rap1, a regulator of neuronal ERK signaling

Over the last decade, it has become well established that ERKs are important in the regulation of processes such as cell proliferation and differentiation. These effects have been best studied in the context of growth factor signaling and accumulating evidence has demonstrated a requirement of ERKs in the actions of growth factors in multiple non-neuronal cell types (Schaeffer and Weber, 1999). However, an emerging theme is that ERK signaling is important in controlling various processes in mature neurons such as gene expression, neuronal excitability and synaptic plasticity (Sweatt, 2001). As such a great deal of attention has focused on the regulatory mechanisms that control ERK signaling in neurons (Grewal, et al., 1999). The experiments presented in this thesis suggest that the small G-protein, Rap1, may be important in controlling the activity of neuronal ERKs. We have demonstrated that calcium influx through depolarization-mediated opening of L-type voltage operated channels can stimulate Rap1:B-Raf signaling pathway in PC12 cells. Like cAMP, activation of this pathway occurs via PKA. Moreover, in PC12 cells, and perhaps hippocampal neurons, activation of this pathway by calcium leads to the stimulation of ERKs. Thus, signaling through Rap1 is a common mechanism by which both calcium influx and cAMP can signal to ERKs in PC12 cells. Furthermore, we also showed that the Rap1-ERK pathway is required for the stimulation of CRE-dependent transcription by both calcium influx and cAMP stimulation in PC12 cells. However, both stimuli use this pathway differently. Calcium influx phosphorylated CREB via a Rap1-ERK dependent pathway. In contrast, cAMP phosphorylated CREB via PKA directly, but required the Rap1-ERK pathway to regulate a component independent of serine-133 phosphorylation in order to achieve full transcription. These data demonstrate that a single pathway can mediate distinct actions on gene transcription depending on the activating stimulus.

The majority of the experiments in this thesis were carried out in the PC12 pheochromocytoma cell line. This cell line has provided an important tool for elucidating neuronal signaling pathways (Kaplan and Stephens, 1994; Marshall, 1995). PC12 cells are responsive to the neurotrophin, NGF, and have proved invaluable in dissecting the various signaling pathways downstream of NGF receptor activation (Chao, 1992). In particular, a great deal of what we know about the regulation of ERK signaling in neurons was originally established by examining the actions of growth factors such as NGF on PC12 cells. Furthermore, PC12 cells are excitable and express the L-type voltage gated ion channel. Therefore, they have been a good model for examining the signal transduction pathways stimulated by calcium influx (Ghosh and Greenberg, 1995).

The results presented in this thesis, taken together with previous findings in our laboratory, demonstrate that three stimuli important for the regulation of neuronal function (neurotrophins, calcium influx and cAMP stimulation) can all activate a Rap1-dependent pathway to regulate ERKs in PC12 cells. Activation of this pathway is important in mediating the physiological actions of these stimuli on gene expression, neurite outgrowth and neuronal differentiation (Grewal, et al., 2000; Grewal, et al., 1999; Vossler, et al., 1997; Yao, et al., 1998; York, et al., 1998). However, a major focus for future work will be the demonstration that the Rap1 pathway can regulate ERK signaling in specific neuronal systems. Perhaps the most important component of this pathway is the Raf isoform, B-Raf. As previously discussed, in the absence of B-Raf, the major effect of Rap1 stimulation is the antagonism of ERK signaling. However, in B-Raf expressing cells, Rap1 can positively couple to ERKs (Vossler, et al., 1997). Results from our laboratory indicate that B-Raf is highly expressed in virtually all neuronal populations examined including dorsal root ganglion cells, motor neurons, hippocampal neurons and cerebellar granule cells((York, et al., 2000) and unpublished observations). Interestingly,

within both the peripheral and central nervous system, B-Raf expression appears to be specifically restricted to neurons. Non-neuronal cells such as astrocytes, Schwann cells and oligodendrocytes do not express B-Raf. This suggests that stimuli that activate Rap1 may have cell-type specific effects on ERK signaling in neuronal versus non-neuronal cells. Given the high expression of B-Raf in neurons, it is very likely that Rap1 activation provides an important route for the regulation of ERKs. Since, our data indicates that a major pathway for Rap1 activation requires PKA, the Rap1:B-Raf pathway provides a mechanism for crosstalk between cAMP/PKA and ERK signaling. This is in contrast to the antagonistic effects of cAMP on ERK activation seen in non-neuronal cells (Cook and McCormick, 1993; Vossler, et al., 1997).

There are a number of examples of physiological actions that are regulated by cAMP signaling through ERKs in neurons. For example, the antiapoptotic effects of cAMP in cerebellar granule cells require activation of ERKs (Villalba, et al., 1997). We have observed a similar requirement for ERK signaling in the ability of cAMP to prevent cell death in PC12 cells induced by trophic factor withdrawal (Grewal, Stork, unpublished data). These actions are consistent with potential role for a Rap1:B-Raf signaling pathway. Current studies in our laboratory are beginning to examine the effect of Rap1 inhibition on these anti-apoptotic actions of cAMP in cerebellar granule and PC12 cells. Studies in *Aplysia* have also demonstrated that the ability of cAMP to activate ERKs is important in the regulation of long-term facilitation of sensory neurons (Martin, et al., 1997). These data argue for a role for a Rap1 pathway, although *Aplysia* homologues of Rap1 and B-Raf have not yet been identified. Similar work in hippocampal neurons also indicates that a PKA-ERK pathway is important in the induction of long-term potentiation (Winder, et al., 1999). Furthermore, unpublished work by Kandel and colleagues demonstrate that transgenic mice expressing an interfering mutant of Rap1, RapN17, exhibit defects in hippocampal LTP (Muzzio, et al., 2000). However, some reports have

disputed the ability of RapN17 to block Rap1 activation (van den Berghe, et al., 1997). In our laboratory, we recently made extensive use of a protein, Rap1GAP1 to effectively antagonize Rap1 signaling (see chapter 4). Current work is being aimed at developing transgenic mice expressing Rap1GAP1. This genetic approach will prove valuable in determining the physiological roles for Rap1 signaling and help reinforce the already emerging view of Rap1 as an important regulator of neuronal function.

It is important to note however, that the results of several studies challenge the notion that B-Raf expression is sufficient for the activation of ERKs by cAMP. For example, rat ciliary ganglion and super cervical ganglion neurons both express B-raf yet do not increase ERK activity in response to cAMP treatment (Creedon, et al., 1996; Meyer-Franke, et al., 1998). Moreover, a recent study reports that Rap1 activation cannot lead to ERK stimulation in a line of PC12 cells (Zwartkruis, et al., 1998). Therefore, the expression of both Rap1 and B-raf within a cell does not necessarily indicate responsivenes of the ERK signaling pathway to cAMP. It is possible that other factor(s) are required. Indeed, a recent paper indicated that the expression levels of 14-3-3 are critical in determining whether cAMP can signal through B-Raf to activate ERKs (Qiu, et al., 2000). There may also be a number of Rap1-independent pathways that couple cAMP to ERK signaling. For example, CNrasGEF is a Ras-specific GEF that can be activated directly by cAMP (Pham, et al., 2000). This activation can lead to the stimulation of ERKs. In addition, PKA can increase ERK activity by inhibiting the actions of two phosphatases, STEP and PTP-SL, that normally function to sequester ERKs in unstimulated cells (Blanco-Aparicio, et al., 1999). It is clear that more studies are required to identify the exact mechanism by which cAMP signaling can activate ERKs in neurons. The availablity of specific molecular antagonists of Rap1, such as RapN17 and Rap1GAP, will be important in directly examining the role of Rap1 in this regulation.

Another area for future work is the identification of the range of stimuli that can activate Rap1 signaling pathway in neurons. As already discussed, three stimuli important in nerve signaling (NGF, cAMP, calcium influx) can regulate Rap1 in the PC12 cell neuronal cell model (Vossler, et al., 1997; Yao, et al., 1998; York, et al., 1998). Whether these stimuli use a similar pathway to regulate neuronal function is unclear. However, one recent study reports that stimulation of dopamine receptors in isolated striatal neuron cultures can activate Rap1 (Zanassi, et al., 2001). In the context of calcium signaling, examining the actions of NMDA receptor stimulation on Rap1 activity will be important. In recent years, many of the effects of NMDA on neuronal excitability and gene expression have been shown to require ERK signaling (Sgambato, et al., 1998; Xia, et al., 1996). However, virtually all studies to date have either shown, or more often, assumed that these actions require Ras. The demonstration that calcium influx through either NMDA or L-type channel can activate Rap1 would suggest an alternate pathway for ERK activation in neurons. Indeed, they may reveal mechanisms for generating signal specificity, with the choice of signal pathway activated (i.e. Ras versus Rap1) being influenced by either the strength or duration of signal input. Alternatively, the existence of two pathways may determine the signaling parameters (e.g. strength/duration) of ERK activation in a manner analogous to EGF and NGF signaling in PC12 cells (Marshall, 1995). There already is evidence that suggests that NMDA receptor activation can influence Rap1 signaling. For example, recent work has identified a RapGAP protein, SpanGAP, which is highly expressed at glutamatergic synapses. SpanGAP was present in a multiprotein complex consisting of the NMDA receptor and PSD95, an anchoring protein with a well-described role in the regulation of post-synaptic signaling. This suggests that activation of the NMDA receptor may be able to influence SpanGAP, and hence Rap1, activity.

How is Rap1 activated ?

A major tenet of this thesis is that Rap1 may be an important regulator of neuronal function in the context of signaling by a diverse array of extracellular signals. An important consideration, therefore is the mechanism by which Rap1 is stimulated. Work presented in this thesis indicates that in the context of calcium signaling this requires PKA. Moreover, previous work in our laboratory indicates that both NGF and cAMP also use a PKA-dependent pathway to stimulate Rap1 (Vossler, et al., 1997; Yao, et al., 1998). However, the exact target of PKA and the mechanism by which Rap1 subsequently becomes activated is still unclear. As already discussed, the activation small G-proteins is stimulated by guanine nucleotide exchange factors (GEFs) which catalyze GTP-loading. Therefore, the regulation of GEF activity may be a target for PKA. For many years and for much of the time that Rap1 has been studied in our laboratory, the only known GEF for Rap1 was C3G (Gotoh, et al., 1995). Evidence in our laboratory suggests C3G may be required for both NGF- and cAMP-mediated Rap1 activation. How, if at all, PKA regulates C3G function is not clear. However, given that GEF activity can be controlled by multiple protein-protein interactions that allow recruitment of GEFs to the membrane, a PKA-dependent phosphorylation event may be important in mediating these events.

In recent years there has been a remarkable increase in the number of Rap1 GEFs that have been identified (see chapter two). Many of these appear to be regulated by a diverse array of intracellular second messengers such as calcium, cAMP and diacylglycerol (de Rooij, et al., 1998; Kawasaki, et al., 1998; Kawasaki, et al., 1998). In addition virtually all these exhibit high expression levels in the brain, including one class that contains a PDZ-domain that may allow targeting to post-synaptic regions (de Rooij, et al., 1999). The identification of these GEFs further emphasizes the role that Rap1 may play as an integrator of multiple signaling pathways. However, the precise mechanism by which these various

GEFs work is not fully clear. Nevertheless, they may contribute to the activation of Rap1 by both calcium and cAMP in neurons. This, it is possible that, in certain circumstances, Rap1 can be stimulated independently of PKA signaling

Rap1 signaling and CREB function

In this thesis, we have demonstrated for the first time that a Rap1-ERK pathway can control CREB phosphorylation by calcium signals. Multiple kinases have been reported to induce CREB phosphorylation in neurons. However, there has been a great deal of debate and a number of apparently contradictory reports about which kinases are responsible for mediating the actions of calcium. Early studies demonstrated a role for CaM kinases, particularly CaM kinase IV (Bito, et al., 1996; Dash, et al., 1991; Deisseroth, et al., 1996; Sheng, et al., 1991; Sun, et al., 1994). More recently, ERK-dependent pathways have been argued to be involved (Impey, et al., 1998; Vanhoutte, et al., 1999). Even here the picture is not entirely clear since two different kinases families (RSKs and MSKs) are capable of phosphorylating CREB in response to ERK activation (Deak, et al., 1998; Impey, et al., 1998; Xing, et al., 1996). To resolve these different findings, a consensus has emerged that the particular calcium-regulated CREB kinase used may be determined by factors such as cell type (e.g. hippocampal vs.cortical), stimulus type (NMDA- vs. L-type channel-induced influx) and stimulus intensity (short vs. prolonged depolarization) (Shaywitz and Greenberg, 1999). Nevertheless, exactly how any one stimulus can selectively stimulate a particular CREB regulatory pathway is still unclear. Examining a role for Rap1 signaling in the regulation of CREB in neurons will therefore depend on identifying both a stimulus and a cell-type in which Rap1 can couple to calcium signaling to ERK activation.

An important result in this thesis was the demonstration that the Rap1-ERK pathway was also required for CREB-mediated transcription by regulating an event downstream of CREB phosphorylation. The target for this action remains to be determined. However, the data presented in chapter four suggest that ERKs may regulate some event downstream of CBP recruitment. These actions may reflect the modulation of CBP activity. For example, a number of studies have demonstrated that GAL4-CBP stimulation of an artificial reporter gene can be controlled by ERK signaling. Although, how ERK phosphorylation regulates CBP activity is unknown. The demonstration that cAMP uses a Rap1-ERK mechanism independent of serine-133 phosphorylation for the regulation of CREB in neurons will be important. This is particularly important in view of the fact that the prevailing dogma is that the PKA dependent phosphorylation of CREB is sufficient to mediate cAMP-dependent regulation of gene expression (Montminy, 1997).

Many physiological effects may potentially be controlled by the Rap1-dependent regulation of CREB-dependent transcription. For example, a number of studies have independently demonstrated that PKA can regulate synaptic plasticity through either ERK or CREB(Impey, et al., 1998; Martin and Kandel, 1996; Martin, et al., 1997; Silva, et al., 1998). The identification of the Rap1 regulatory pathway indicates that some of these actions may reflect a linear PKA-ERK-CREB signaling cascade. The target genes that may be regulated by CREB in this context are unclear. However, the calcium-dependent expression neurotrophins such as BDNF requires signaling through CREB and may contribute to activity-dependent increases synaptic function (Shieh, et al., 1998; Tao, et al., 1998). Moreover, cAMP can increase the expression of a number of neuropeptides through CRE-dependent transcription (Goodman, 1990). The emerging use of cDNA array technology will undoubtedly expand our knowledge of CREB-dependent target genes and in particular will help identify genes that are unregulated through distinct pathways (e.g. calcium versus cAMP; PKA versus ERKs).

The Rap1-CREB pathway may also regulate neuronal survival. As discussed, the ability of cAMP to promote the survival of various neuronal populations such a cerebellar granule cells, may be mediated through a Rap1-Erk pathway (Villalba, et al., 1997). Moreover, recent studies have suggested that CREB-dependent gene expression might be important to promote neuron survival (Bonni, et al., 1999; Riccio, et al., 1999). In particular, constitutive stimulation of CRE-dependent transcription in both cerebellar granule cells and sympathetic neurons was sufficient to protect against cell death induced by trophic factor-withdrawal . The up-regulated expression of the prosurvival protein, BCL-2, was identified as one target of CREB in mediating these anti-apoptotic actions (Riccio, et al., 1999). These CREB survival effects were described in the context of neurotrophin signaling. However, cAMP may also use a similar mechanism to mediate cell survival by regulating CREB function through the activation of the Rap1-ERK pathway.

As well as promoting cell survival, cAMP can also induce the growth arrest of certain mitotically active neuronal precursors (Lu and DiCicco-Bloom, 1997). Similarly, cAMP can block the proliferation of PC12 cells (Farinelli and Greene, 1996; Mark and Storm, 1997). In fact, these antiproliferative effects are thought to account for the ability of cAMP to synergize with EGF to induce neuronal differentiation in PC12 cells (Mark, et al., 1995). The mechanism by which cAMP induces these anti-mitotic effects is unclear. However, several lines of evidence suggest a potential role for both ERKs and CREB. For example, work in our laboratory and elsewhere has shown that the synergy between cAMP and EGF requires the ability of cAMP to stimulate ERKS and is associated with an increase in CREB phosphorylation (Mark, et al., 1995; Mark and Storm, 1997; Yao, et al., 1995). Moreover, one recent report has demonstrated that the cAMP-induced growth arrest within a particular population of cortical neuronal precursors was preceded by a selective increase in CREB phosphorylation within these cells. The availability of specific molecular

inhibitors of both Rap1 and CREB will allow us to directly test the hypothesis that they are required for either the antiprolifertive or survival effects of cAMP in both neurons and PC12 cells.

Signal specificity within neurons

One notable and quite interesting finding in this thesis was that, while Rap1-ERK signaling was required for CREB regulation, it was used differently by both calcium and cAMP. This illustrates a recurring enigmatic question in signal transduction, namely how can activation of the same signaling pathway by different stimuli lead to distinct cellular outcomes (Marshall, 1995). For example, why does activation of PKA in one case (cAMP stimulation) phosphorylate CREB directly, while in another (calcium influx), the effects on CREB require an indirect action via a Rap1-ERK pathway ? A likely answer is that the two stimuli activate spatially distinct 'pools' of PKA. Thus, G protein-coupled receptor activation and forskolin stimulation may induce a global increase in cAMP signal that is sufficient to activate multiple pools of PKA leading to both nuclear translocation of PKA and stimulation of the Rap1-ERK pathway. In contrast, the actions of calcium influx may be limited to a more discreet, localized increase in PKA activation that may efficiently couple to Rap1-ERK activation, but cannot lead to a robust nuclear translocation and direct phosphorylation of CREB. These distinct actions may be achieved by the anchoring of PKA via specific AKAPs (Colledge and Scott, 1999). For example, plasma membrane associated AKAPs may be important in facilitating the PKA-dependent activation of Rap1. In contrast, an AKAP such as mAKAP, which targets PKA to a perinuclear localization, may be important in regulating the nuclear actions of PKA on CREB phosphorylation. Central to this model of PKA signaling is the notion that the actions of kinase signaling pathways are determined in large part by the subcellular location within a cell that they become stimulated. This, in fact, is an emerging theme in signal transduction (Hunter,

2000; Pawson, 1995; Pawson and Scott, 1997). Research over the last three decades has revealed the existence of many kinase signaling pathways. In particular, this work has catalogued an almost bewildering level of crosstalk between these pathways (Hunter, 2000). However, understanding how they signal at the subcellular level will probably give us greater insight into the mechanism by which they regulate various cellular events.

Given this emphasis on spatial and subcellular signaling, it is interesting to consider the regulation of signaling events in neurons, perhaps the most polarized of all cells. In particular we can examine the mechanism by which PKA regulates CREB. Based on the findings reported in this thesis, two potential pathways exist for such regulation. One is the direct nuclear translocation and phosphorylation of CREB by PKA. The alternative pathway involves the indirect stimulation of Rap1 leading to ERK-dependent phosphorylation of CREB. What factors might influence which of these pathways contributes to the regulation of CREB? One possibility is that the robust stimulation of cAMP signaling within the cell body may be sufficient to lead to nuclear localization of PKA and stimulation of the direct pathway. In contrast, the discrete stimulation of cAMP, for example at post-synaptic sites along dendrites or at axon terminals, may lead to the PKA-dependent activation of Rap1. This may subsequently induce a retrograde ERK signal that is propagated back to the cell body to regulate CREB. This pathway may depend on the formation of retrogradely transported signaling vesicles (see chapter two and Figure 5.1).

A major challenge is to begin to characterize these spatially regulated signaling pathways. One approach is to try and selectively block signaling within distinct cellular compartments. An example of such an approach was use by Bading and colleagues to examine calcium-dependent regulation of gene expression (Hardingham, et al., 1997). They made use of a modified form of BAPTA, a calcium chelator. Incubation of cells with BAPTA inhibits

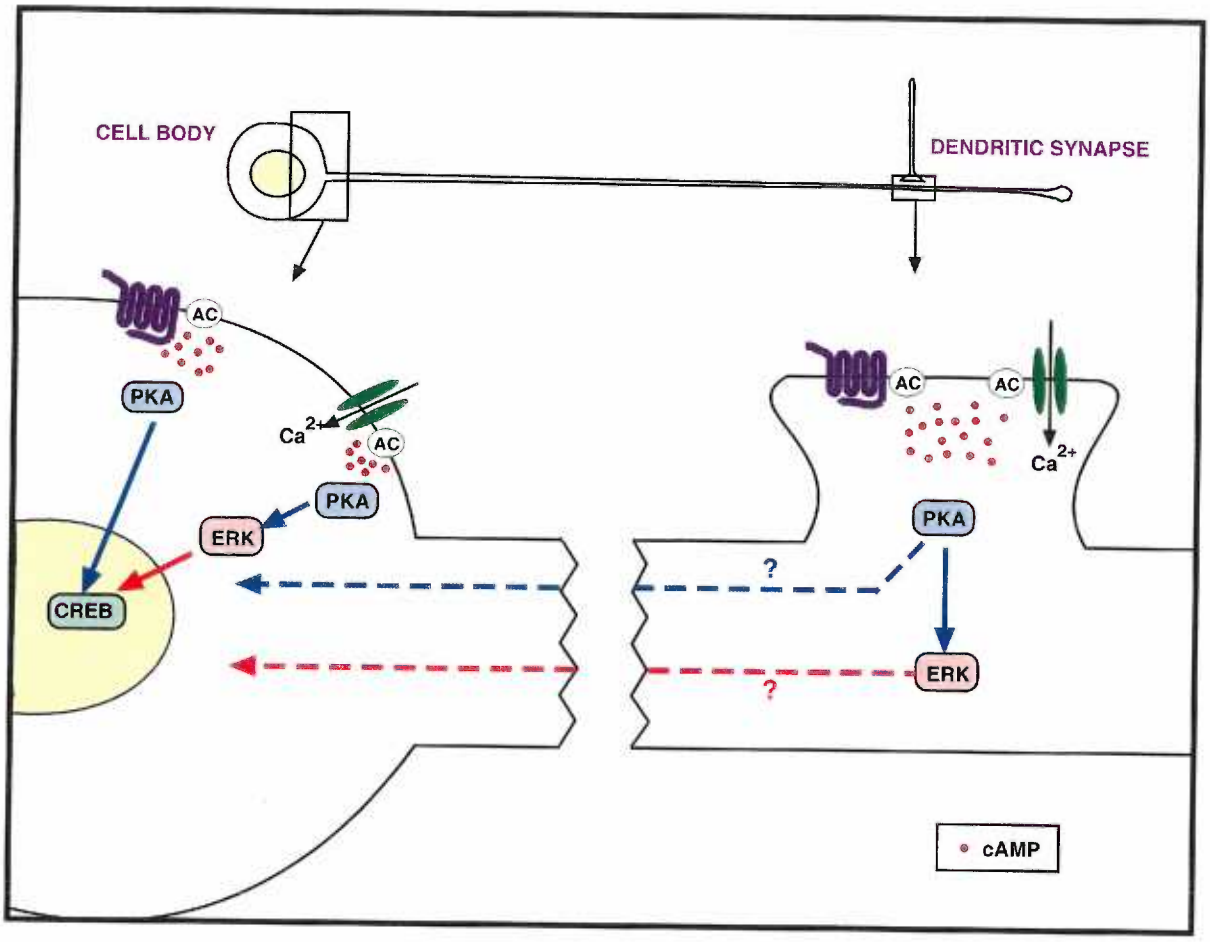

Figure 5.1. **PKA and ERK signaling in neurons.** The actions and regulation of PKA/ERK signaling may depend on subcellular localization. For example, stimulation of cAMP by G-protein-coupled receptors in the cell body may allow for direct translocation of PKA into the nucleus and phosphorylation of CREB. In contrast, the stimulation of similar receptors at postsynaptic dendritic sites may lead to only a local increase in cAMP/PKA signaling. While this may not be sufficient for nuclear translocation, it may stimulate a retrograde ERK signal that can lead to CREB phosphorylation. This PKA-ERK-CREB pathway, may also be stimulated by local increases in cAMP levels mediated by calcium influx in either the cell body or dendrites.

calcium signaling in all parts of the cell. However, these workers also injected BAPTA coupled to a 70K-dextran molecule into the nucleus. This prevents BAPTA from diffusing into the cytoplasm and therefore specifically blocks nuclear calcium. Using this approach, they were able to distinguish between the requirements for nuclear versus cytoplasmic calcium in the regulation of gene expression (Hardingham, et al., 1997). A similar approach could be used to selectively inhibit kinase signaling pathways. For example, interfering mutants fused to targeting motifs (e.g. nuclear localization sequences, myristolation sequences etc.) could be generated to inhibit kinase action at distinct subcellular sites (e.g. nucleus, membrane etc.).

An alternate approach could use Campenot chambers to study neuronal signaling. This elegant culture system allows either sympathetic or sensory neurons to be grown in multi-chamber dishes in which different factors and pharmacological stimulators or inhibitors canbe applied to distinct regions of the neurons (Campenot, 1994; Kuruvilla, et al., 2000; Riccio, et al., 1997; Senger and Campenot, 1997). Thus, this system could be used to examine how inhibition of either ERKs or PKA within cell bodies or axon terminals affects the ability of cAMP, applied to either cell bodies or nerve terminals, to stimulate CREB and CREB-dependent transcription. For example, if discrete stimulation of cAMP within neurites uses a Rap1-ERK pathway to phosphorylate CREB, then inhibition of PKA within cell bodies would have no effect on this response. Examining the actions of both calcium and cAMP using Campenot chambers therefore provides a simple, yet powerful, way to begin to understand the effects of localized signaling in neurons.

In conclusion, the major goal of this thesis was to further our understanding of Rap1-dependent signaling. In this context, we have established that the Rap1 pathway represents an important target for calcium signaling to ERKs. Moreover, activation of Rap1 signaling is important in the regulation of CREB transcription. We hope and anticipate that these

findings will provide strong impetus for future studies examining Rap1 function in neurons.

REFERENCES

Abel, T., P. V. Nguyen, M. Barad, T. A. Deuel, E. R. Kandel, and R. Bourtchouladze. 1997. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 88:615-626.

Adams, J. P., A. E. Anderson, A. W. Varga, K. T. Dineley, R. G. Cook, P. J. Pfaffinger, and J. D. Sweatt. 2000. The A-type potassium channel kv4.2 is a substrate for the mitogen-activated protein kinase ERK. J. Neurochem. 75:2277-87.

Agnihotri, N., W. S. Kisaalita, and C. H. Keith. 1997. Free cyclic AMP increases in PC12 cells on depolarization. J. Neurosci. Res. 47:555-60.

Ait-Si-Ali, S., D. Carlisi, S. Ramirez, L. C. Upegui-Gonzalez, A. Duquet, P. Robin, B. Rudkin, A. Harel-Bellan, and D. Trouche. 1999. Phosphorylation by p44 MAP Kinase/ERK1 stimulates CBP histone acetyl transferase activity in vitro. Biochem. Biophys. Res. Commun. 262:157-62.

Alberini, C. M., M. Ghirardi, R. Metz, and E. R. Kandel. 1994. C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in Aplysia. Cell 76:1099-114.

Anderson, A. E., J. P. Adams, J. W. Swann, Jognston, D, P. J. Pfaffinger, and J. D. Sweatt. 1998. Kv4.2, a transient A-type K+ channel, is a substrate for multiple kinases involved in LTP Induction. Soc. Neurosci. Abstr. :9.4.

Anderson, C. N. G., and A. M. Tolkovsky. 1999. A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. J. Neurosci. 19:664-73.

Arias, J., A. S. Alberts, P. Brindle, F. X. Claret, T. Smeal, M. Karin, J. Feramisco, and M. Montminy. 1994. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor [see comments]. Nature 370:226-9.

Artalejo, C. R. 1997. More on calcium currents. Trends Neurosci. 20:448-50.

Atkins, C. M., J. C. Selcher, J. J. Petraitis, J. M. Trzaskos, and J. D. Sweatt. 1998. The MAPK cascade is required for mammalian associative learning. Nat. Neurosci. 1:602-9.

Bading, H., D. D. Ginty, and M. E. Greenberg. 1993. Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science 260:181-6.

Bailey, C. H., D. Bartsch, and E. R. Kandel. 1996. Toward a molecular definition of long-term memory storage. Proc. Natl. Acad. Sci. USA 93:13445-13452.

Bailey, C. H., B. K. Kaang, M. Chen, K. C. Martin, C. S. Lim, A. Casadio, and E. R. Kandel. 1997. Mutation in the phosphorylation sites of MAP kinase blocks learning- related internalization of apCAM in Aplysia sensory neurons [see comments]. Neuron 18:913-24.

Bannister, A. J., and T. Kouzarides. 1996. The CBP co-activator is a histone acetyltransferase. Nature 384:641-3.

Bar-Sagi, D., and A. Hall. 2000. Ras and Rho GTPases: a family reunion. Cell 103:227-38.

Bergmann, A., J. Agapite, K. McCall, and H. Steller. 1998. The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling [see comments]. Cell 95:331-41.

Berman, D. E., S. Hazvi, K. Rosenblum, R. Seger, and Y. Dudai. 1998. Specific and differential activation of mitogen-activated protein kinase cascades by unfamiliar taste in the insular cortex of the behaving rat. J. Neurosci. 18:10037-44.

Berridge, M. J. 1998. Neuronal calcium signaling. Neuron 21:13-26.

Bhat, R. V., T. M. Engber, J. P. Finn, K. J. Koury, P. C. Contreras, M. S. Miller, D. A. Dionne, and K. M. Walton. 1998. Region-specific targets of p42/p44MAPK signaling in rat brain. J. Neurochem. 70:558-571.

Bito, H. 1998. The role of calcium in activity-dependent neuronal gene regulation. Cell Calcium 23:143-50.

Bito, H., K. Deisseroth, and R. W. Tsien. 1997. Ca2+-dependent regulation in neuronal gene expression. Curr. Opin. Neurobiol. 7:419-29.

Bito, H., K. Deisseroth, and R. W. Tsien. 1996. CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87:1203-14.

Blanco-Aparicio, C., J. Torres, and R. Pulido. 1999. A novel regulatory mechanism of MAP kinases activation and nuclear translocation mediated by PKA and the PTP-SL tyrosine phosphatase. J. Cell Biol. 147:1129-36.

Blitzer, R. D., T. Wong, R. Nouranifar, R. Iyengar, and E. M. Landau. 1995. Postsynaptic cAMP pathway gates early LPT in hippocampal CA1 region. Neuron 15:1403-1414.

Blum, S., A. N. Moore, F. Adams, and P. K. Dash. 1999. A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. J. Neurosci. 19:3535-44.

Bonni, A., A. Brunet, A. E. West, S. R. Datta, M. A. Takasu, and M. E. Greenberg. 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286:1358-62.

Bonni, A., D. D. Ginty, H. Dudek, and M. E. Greenberg. 1995. Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. Mol. Cell. Neurosci. 6:168-83.

Bos, J. L. 1998. All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. EMBO J. 17:6776-82.

Boussiotis, V. A., G. J. Freeman, A. Berezovskaya, D. L. Barber, and L. M. Nadler. 1997. Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. Science 278:124-8.

Brambilla, R., N. Gnesutta, L. Minichiello, G. White, A. J. Roylance, C. E. Herron, M. Ramsey, D. P. Wolfer, V. Cestari, C. Rossi-Arnaud, S. G. Grant, P. F. Chapman, H. P. Lipp, E. Sturani, and R. Klein. 1997. A role for the Ras signalling pathway in synaptic transmission and long- term memory. Nature 390:281-6.

Brenman, J. E., D. S. Chao, S. H. Gee, A. W. McGee, S. E. Craven, D. R. Santillano, Z. Wu, F. Huang, H. Xia, M. F. Peters, S. C. Froehner, and D. S. Bredt. 1996. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell 84:757-67.

Briggs, L. J., D. Stein, J. Goltz, V. C. Corrigan, A. Efthymiadis, S. Hubner, and D. A. Jans. 1998. The cAMP-dependent protein kinase site (Ser312) enhances dorsal nuclear import through facilitating nuclear localization sequence/importin interaction. J. Biol. Chem. 273:22745-52.

Brunet, A., D. Roux, P. Lenormand, S. Dowd, S. Keyse, and J. Pouyssegur. 1999. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J. 18:664-74.

Brunner, D., K. Ducker, N. Oellers, E. Hafen, H. Scholz, and C. Klambt. 1994. The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. Nature 370:386-9.

Brunner, D., N. Oellers, J. Szabad, W. H. BiggssIII, S. L. Zipursky, and E. Hafen. 1994. A gain-of function mutation in drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell 76:875-888.

Buonanno, A., and R. D. Fields. 1999. Gene regulation by patterned electrical activity during neural and skeletal muscle development. Curr. Opin. Neurobiol. 9:110-20.

Burgering, B. M. T., and J. L. Bos. 1995. Regulation of Ras-mediated signalling: more than one way to skin a cat. TIBS 20:18-22.

Cain, D. P. 1997. LTP, NMDA, genes and learning. Curr. Opin. Neurobiol. 7:235-42.

Campenot, R. B. 1994. NGF and the local control of nerve terminal growth. J. Neurobiol. 25:599-611.

Canagarajah, B. J., A. Khokhlatchev, M. H. Cobb, and E. J. Goldsmith. 1997. Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell 90:859-69.

Cardinaux, J. R., J. C. Notis, Q. Zhang, N. Vo, J. C. Craig, D. M. Fass, R. G. Brennan, and R. H. Goodman. 2000. Recruitment of CREB binding protein is sufficient for CREB-mediated gene activation. Mol. Cell. Biol. 20:1546-52.

Carr, D. W., R. E. Stofko-Hahn, I. D. Fraser, R. D. Cone, and J. D. Scott. 1992. Localization of the cAMP-dependent protein kinase to the postsynaptic densities by A-kinase anchoring proteins. Characterization of AKAP 79. J. Biol. Chem. 267:16816-23.

Chao, M. V. 1992. Growth factor signaling: Where is the specificity. Cell 68:995-997.

Chawla, S., G. E. Hardingham, D. R. Quinn, and H. Bading. 1998. CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. Science 281:1505-9.

Chen, H. J., M. Rojas-Soto, A. Oguni, and M. B. Kennedy. 1998. A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. Neuron 20:895-904.

Chen, W., T. S. Shields, P. J. S. Stork, and R. D. Cone. 1995. A colorimetric assay for measuring activation of G_s- and G_q-coupled signaling pathways. Analytical Biochem. 226:349-354.

Chijiwa, T., A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, and H. Hidaka. 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor

of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J. Biol. Chem. 265:5267-72.

Childs, T. J., M. H. Watson, J. S. Sanghera, D. L. Campbell, S. L. Pelech, and A. S. Mak. 1992. Phosphorylation of smooth muscle caldesmon by mitogen-activated protein (MAP) kinase and expression of MAP kinase in differentiated smooth muscle cells. J. Biol. Chem. 267:22853-9.

Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy, and R. H. Goodman. 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365:855-9.

Chung, J., E. Uchida, T. C. Grammer, and J. Blenis. 1997. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol. Cell. Biol. 17:6508-16.

Clapham, D. E. 1995. Calcium signaling. Cell 80:259-68.

Colangelo, A. M., P. F. Johnson, and I. Mocchetti. 1998. beta-adrenergic receptor-induced activation of nerve growth factor gene transcription in rat cerebral cortex involves CCAAT/enhancer-binding protein delta. Proc. Natl. Acad. Sci. USA 95:10920-5.

Colledge, M., R. A. Dean, G. K. Scott, L. K. Langeberg, R. L. Huganir, and J. D. Scott. 2000. Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. Neuron 27:107-19.

Colledge, M., and J. D. Scott. 1999. AKAPs: from structure to function. Trends Cell Biol. 9:216-21.

Coogan, A. N., D. M. O'Leary, and J. J. O'Connor. 1999. P42/44 MAP kinase inhibitor PD98059 attenuates multiple forms of synaptic plasticity in rat dentate gyrus in vitro. J Neurophysiol 81:103-10.

Cook, S. J., and F. McCormick. 1993. Inhibition by cAMP of Ras-dependent activation of Raf. Science 262:1069-72.

Cook, S. J., B. Rubinfeld, I. Albert, and F. McCormick. 1993. RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. EMBO J. 12:3475-85.

Crabtree, G. R. 1999. Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. Cell 96:611-4.

Creedon, D. J., J. E. M. Johnson, and J. J. C. Lawrence. 1996. Mitogen-activated protein kinase-independent pathways mediate the effects of nerve growth factor and cAMP on neuronal survival. J. Biol. Chem. 271:20713-20718.

Crews, C. M., A. Alessandrini, and R. L. Erikson. 1992. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science 258:478-80.

Crews, C. M., and R. L. Erikson. 1993. Extracellular signals and reversible protein phosphorylation: what to Mek of it all. Cell 74:215-7.

Crow, T., J. J. Xue-Bian, V. Siddiqi, Y. Kang, and J. T. Neary. 1998. Phosphorylation of mitogen-activated protein kinase by one-trial and multi-trial classical conditioning. J. Neurosci. 18:3480-7.

Crowder, R. J., and R. S. Freeman. 1999. The survival of sympathetic neurons promoted by potassium depolarization, but not by cyclic AMP, requires phosphatidylinositol 3-kinase and Akt. J. Neurochem. 73:466-75.

Cruzalegui, F. H., G. E. Hardingham, and H. Bading. 1999. c-Jun functions as a calcium-regulated transcriptional activator in the absence of JNK/SAPK1 activation. EMBO J. 18:1335-44.

Curran, T., and J. I. Morgan. 1995. Fos: an immediate-early transcription factor in neurons. J. Neurobiol. 26:403-12.

Daaka, Y., L. M. Luttrell, S. Ahn, G. J. Della Rocca, S. S. Ferguson, M. G. Caron, and R. J. Lefkowitz. 1998. Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J. Biol. Chem. 273:685-8.

Dalby, K. N., N. Morrice, F. B. Caudwell, J. Avruch, and P. Cohen. 1998. Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. J. Biol. Chem. 273:1496-505.

Daniel, P. B., W. H. Walker, and J. F. Habener. 1998. Cyclic AMP signaling and gene regulation. Annu Rev Nutr 18:353-83.

Dash, P. K., K. A. Karl, M. A. Colicos, R. Prywes, and E. R. Kandel. 1991. cAMP response element-binding protein is activated by Ca2+/calmodulin- as well as cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA 88:5061-5.

Davis, R. J. 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 268:14553-14556.

de Rooij, J., N. M. Boenink, M. van Triest, R. H. Cool, A. Wittinghofer, and J. L. Bos. 1999. PDZ-GEF1, a guanine nucleotide exchange factor specific for Rap1 and Rap2. J. Biol. Chem. 274:38125-30.

de Rooij, J., F. J. Zwartkruis, M. H. Verheijen, R. H. Cool, S. M. Nijman, A. Wittinghofer, and J. L. Bos. 1998. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP [see comments]. Nature 396:474-7.

Deak, M., A. D. Clifton, L. M. Lucocq, and D. R. Alessi. 1998. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J. 17:4426-41.

Deisseroth, K., H. Bito, and R. W. Tsien. 1996. Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron 16:89-101.

Della Fazia, M. A., G. Servillo, and P. Sassone-Corsi. 1997. Cyclic AMP signalling and cellular proliferation: regulation of CREB and CREM. FEBS Lett. 410:22-4.

Della Rocca, G. J., Y. V. Mukhin, M. N. Garnovskaya, Y. Daaka, G. J. Clark, L. M. Luttrell, R. J. Lefkowitz, and J. R. Raymond. 1999. Serotonin 5-HT1A receptor-mediated Erk activation requires calcium/calmodulin-dependent receptor endocytosis. J. Biol. Chem. 274:4749-53.

DeManno, D. A., J. E. Cottom, M. P. Kline, C. A. Peters, E. T. Maizels, and M. Hunzicker-Dunn. 1999. Follicle-stimulating hormone promotes histone H3 phosphorylation on serine-10. Mol. Endocrinol. 13:91-105.

Dent, P., W. Haser, T. A. Haystead, L. A. Vincent, T. M. Roberts, and T. W. Sturgill. 1992. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science 257:1404-7.

Downward, J. 1995. KSR: a novel player in the RAS pathway. Cell 83:831-4.

Duffy, J. B., and N. Perrimon. 1996. Recent advances in understanding signal transduction pathways in worms and flies. Curr. Opin. Cell. Biol. 8:231-8.

Duffy, J. B., and N. Perrimon. 1994. The torso pathway in Drosophila: lessons on receptor tyrosine kinase signaling and pattern formation. Dev. Biol. 166:380-95.

Dugan, L. L., J. S. Kim, Y. Zhang, R. D. Bart, Y. Sun, D. M. Holtzman, and D. H. Gutmann. 1999. Differential effects of cAMP in neurons and astrocytes. Role of B-raf. J. Biol. Chem. 274:25842-8.

Dumont, J. E., J. C. Jauniaux, and P. P. Roger. 1989. The cyclic AMP-mediated stimulation of cell proliferation. Trends Biochem. Sci. 14:67-71.

Ebinu, J. O., D. A. Bottorff, E. Y. Chan, S. L. Stang, R. J. Dunn, and J. C. Stone. 1998. RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. Science 280:1082-6.

Egea, J., C. Espinet, and J. X. Comella. 1999. Calcium influx activates extracellular-regulated kinase/mitogen- activated protein kinase pathway through a calmodulin-sensitive mechanism in PC12 cells. J. Biol. Chem. 274:75-85.

Engh, R. A., A. Girod, V. Kinzel, R. Huber, and D. Bossemeyer. 1996. Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. Structural implications for selectivity. J. Biol. Chem. 271:26157-64.

English, J. D., and J. D. Sweatt. 1996. Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. J. Biol. Chem. 271:24329-32.

English, J. D., and J. D. Sweatt. 1997. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J. Biol. Chem. 272:19103-6.

Epstein, C. J., L. J. de Asua, and E. Rozengurt. 1975. The role of cyclic AMP in myogenesis. J. Cell. Physiol. 86:83-90.

Erickson, A. K., D. M. Payne, P. A. Martino, A. J. Rossomando, J. Shabanowitz, M. J. Weber, D. F. Hunt, and T. W. Sturgill. 1990. Identification by mass spectrometry of threonine 97 in bovine myelin basic protein as a specific phosphorylation site for mitogen-activated protein kinase. J. Biol. Chem. 265:19728-35.

Fantl, W. J., A. J. Muslin, A. Kikuchi, J. A. Martin, A. M. MacNicol, R. W. Gross, and L. T. Williams. 1994. Activation of Raf-1 by 14-3-3 proteins. Nature 371:612-4.

Farinelli, S. E., and L. A. Greene. 1996. Cell cycle blockers mimosine, ciclopirox, and deferoxamine prevent the death of PC12 cells and postmitotic sympathetic neurons after removal of trophic support. J. Neurosci. 16:1150-1162.

Farnsworth, C. L., N. W. Freshney, L. B. Rosen, A. Ghosh, M. E. Greenberg, and L. A. Feig. 1995. Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. Nature 376:524-7.

Feng, X. H., Y. Zhang, R. Y. Wu, and R. Derynck. 1998. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. Genes Dev. 12:2153-63.

Ferrigno, P., F. Posas, D. Koepp, H. Saito, and P. A. Silver. 1998. Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. EMBO J. 17:5606-14.

Fields, R. D., F. Eshete, B. Stevens, and K. Itoh. 1997. Action potential-dependent regulation of gene expression: temporal specificity in ca2+, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. J. Neurosci. 17:7252-66.

Finkbeiner, S., and M. E. Greenberg. 1996. Ca(2+)-dependent routes to Ras: mechanisms for neuronal survival, differentiation, and plasticity? Neuron 16:233-6.

Fiol, C. J., J. S. Williams, C. H. Chou, Q. M. Wang, P. J. Roach, and O. M. Andrisani. 1994. A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression. J. Biol. Chem. 269:32187-93.

Fiore, R. S., T. H. Murphy, J. S. Sanghera, S. L. Pelech, and J. M. Baraban. 1993. Activation of mitogen-activated protein kinase by glutamate receptor stimulation in rat primary cortical cultures. J. Neurochem. 61:1626-1633.

Franklin, J. A., and E. M. Johnson, Jr. 1992. Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. Trends Neurosci. 15:501-508.

Fraser, I. D., and J. D. Scott. 1999. Modulation of ion channels: a "current" view of AKAPs. Neuron 23:423-6.

Frey, U., Y.-Y. Huang, and E. R. Kandel. 1993. Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. Science 260:1661-1664.

Fukunaga, R., and T. Hunter. 1997. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J. 16:1921-33.

Gadbois, D. M., H. A. Crissman, R. A. Tobey, and E. M. Bradbury. 1992. Multiple kinase arrest points in the G1 phase of nontransformed mammalian cells are absent in transformed cells. Proc. Natl. Acad. Sci. USA 89:8626-30.

Gallin, W. J., and M. E. Greenberg. 1995. Calcium regulation of gene expression in neurons: the mode of entry matters. Curr. Opin. Neurobiol. 5:367-74.

Galter, D., and K. Unsicker. 2000. Brain-derived neurotrophic factor and trkB are essential for cAMP-mediated induction of the serotonergic neuronal phenotype. J. Neurosci. Res. 61:295-301.

Ghosh, A., D. D. Ginty, H. Bading, and M. E. Greenberg. 1994. Calcium regulation of gene expression in neuronal cells. J. Neurobiol. 25:294-303.

Ghosh, A., and M. E. Greenberg. 1995. Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science 268:239-47.

Ginty, D. D. 1997. Calcium regulation of gene expression: isn't that spatial? Neuron 18:183-6.

Ginty, D. D., D. Glowacka, D. S. Bader, H. Hidaka, and J. A. Wagner. 1991. Induction of immediate early genes by Ca2+ influx requires cAMP- dependent protein kinase in PC12 cells. J. Biol. Chem. 266:17454-8.

Ginty, D. D., J. M. Kornhauser, M. A. Thompson, H. Bading, K. E. Mayo, J. S. Takahashi, and M. E. Greenberg. 1993. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science 260:238-41.

Goelet, P., V. F. Castellucci, S. Schacher, and E. R. Kandel. 1986. The long and the short of long-term memory-a molecular frame-work. Nature 322:419-422.

Goldberg, J. L., and B. A. Barres. 2000. The relationship between neuronal survival and regeneration. Annu. Rev. Neurosci. 23:579-612.

Gonzalez, G. A., and M. R. Montminy. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675-80.

Goodman, R. H. 1990. Regulation of neuropeptide gene expression. Annu. Rev. Neurosci. 13:111-27.

Goslin, K., and G. Banker. 1991. Rat hippocampal neurons in low-density culture. In: Culturing nerve cells. Cambridge: MIT.

Gotoh, T., S. Hattori, S. Nakamura, H. Kitayama, M. Noda, Y. Takai, K. Kaibuchi, H. Matsui, O. Hatase, H. Takahashi, and et al. 1995. Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. Mol. Cell. Biol. 15:6746-53.

Graef, I. A., P. G. Mermelstein, K. Stankunas, J. R. Neilson, K. Deisseroth, R. W. Tsien, and G. R. Crabtree. 1999. L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. Nature 401:703-8.

Greenberg, M. E., E. B. Ziff, and L. A. Greene. 1986. Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. Science 234:80-3.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA 73:2424-8.

Grewal, S. S., A. M. Horgan, R. D. York, G. S. Withers, G. A. Banker, and P. J. Stork. 2000. Neuronal calcium activates a Rap1 and B-Raf signaling pathway via the cyclic adenosine monophosphate-dependent protein kinase. J. Biol. Chem. 275:3722-8.

Grewal, S. S., R. D. York, and P. J. Stork. 1999. Extracellular-signal-regulated kinase signalling in neurons. Curr. Opin. Neurobiol. 9:544-553.

Grimes, M. L., E. Beattie, and W. C. Mobley. 1997. A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. Proc. Natl. Acad. Sci. USA 94:9909-14.

Gundersen, G. G., and T. A. Cook. 1999. Microtubules and signal transduction. Curr. Opin. Cell. Biol. 11:81-94.

Gurd, J. W., and N. Bissoon. 1997. The N-methyl-D-aspartate receptor subunits NR2A and NR2B bind to the SH2 domains of phospholipase C-gamma. J. Neurochem. 69:623-30.

Hadari, Y. R., H. Kouhara, I. Lax, and J. Schlessinger. 1998. Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. Mol. Cell. Biol. 18:3966-73.

Hagiwara, M., A. Alberts, P. Brindle, J. Meinkoth, J. Feramisco, T. Deng, M. Karin, S. Shenolikar, and M. Montminy. 1992. Transcriptional attenuation following cAMP induction requires PP-1- mediated dephosphorylation of CREB. Cell 70:105-13.

Hagiwara, M., P. Brindle, A. Harootunian, R. Armstrong, J. Rivier, W. Vale, R. Tsien, and M. R. Montminy. 1993. Coupling of hormonal stimulation and transcription via the cyclic AMP- responsive factor CREB is rate limited by nuclear entry of protein kinase A. Mol. Cell. Biol. 13:4852-9.

Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marshall. 1989. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell 57:1167-77.

Hancock, J. F., H. Paterson, and C. J. Marshall. 1990. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. Cell 63:133-9.

Hardingham, G. E., S. Chawla, F. H. Cruzalegui, and H. Bading. 1999. Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. Neuron 22:789-98.

Hardingham, G. E., S. Chawla, C. M. Johnson, and H. Bading. 1997. Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. Nature 385:260-5.

Hayashi, T., H. Umemori, M. Mishina, and T. Yamamoto. 1999. The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. Nature 397:72-6.

Haycock, J. W., N. G. Ahn, M. H. Cobb, and E. G. Krebs. 1992. ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. Proc. Natl. Acad. Sci. USA 89:2365-9.

Herskowitz, I. 1995. MAP kinase pathways in yeast: for mating and more. Cell 80:187-97.

Hidaka, H., and T. Tanaka. 1983. Naphthalenesulfonamides as calmodulin antagonists. Methods Enzymol. 102:185-94.

Hoffmann, R., G. S. Baillie, S. J. MacKenzie, S. J. Yarwood, and M. D. Houslay. 1999. The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. EMBO J. 18:893-903.

Holland, P. M., and J. A. Cooper. 1999. Protein modification: docking sites for kinases. Curr. Biol. 9:R329-31.

Houslay, M. D., and G. Milligan. 1997. Tailoring cAMP-signalling responses through isoform multiplicity. Trends Biochem. Sci. 22:217-224.

Hu, S. C., J. Chrivia, and A. Ghosh. 1999. Regulation of CBP-mediated transcription by neuronal calcium signaling. Neuron 22:799-808.

Huang, Y.-Y., and E. R. Kandel. 1998. Postsynaptic induction and PKA-dependent expression of LTP in the lateral amygdala. Neuron 21:169-178.

Hunter, T. 2000. Signaling--2000 and beyond. Cell 100:113-27.

Ignatova, E. G., M. M. Belcheva, L. M. Bohn, M. C. Neuman, and C. J. Coscia. 1999. Requirement of receptor internalization for opioid stimulation of mitogen-activated protein kinase: biochemical and immunofluorescence confocal microscopic evidence. J. Neurosci. 19:56-63.

Impey, S., M. Mark, E. C. Villacres, S. Poser, C. Chavkin, and D. R. Storm. 1996. Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron 16:973-82.

Impey, S., K. Obrietan, and D. Storm. 1999. Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. Neuron 23:11-14.

Impey, S., K. Obrietan, S. T. Wong, S. Poser, S. Yano, G. Wayman, J. C. Deloulme, G. Chan, and D. R. Storm. 1998. Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB- dependent transcription and ERK nuclear translocation. Neuron 21:869-83.

Impey, S., D. M. Smith, K. Obrietan, R. Donahue, C. Wade, and D. R. Storm. 1998. Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning [see comments]. Nat. Neurosci. 1:595-601.

Impey, S., G. Wayman, Z. Wu, and D. R. Storm. 1994. Type I adenylyl cyclase functions as a coincidence detector for control of cyclic AMP response element-mediated transcription: synergistic regulation of transcription by Ca^{2+} and isoproterenol. Mol. Cell. Biol. 14:8272-8281.

Ishimaru, S., R. Williams, E. Clark, H. Hanafusa, and U. Gaul. 1999. Activation of the Drosophila C3G leads to cell fate changes and overproliferation during development, mediated by the RAS-MAPK pathway and RAP1. EMBO J. 18:145-55.

Janknecht, R. 1996. Analysis of the ERK-stimulated ETS transcription factor ER81. Mol. Cell. Biol. 16:1550-6.

Janknecht, R., D. Monte, J. L. Baert, and Y. de Launoit. 1996. The ETS-related transcription factor ERM is a nuclear target of signaling cascades involving MAPK and PKA. Oncogene 13:1745-54.

Janknecht, R., and A. Nordheim. 1996. MAP kinase-dependent transcriptional coactivation by Elk-1 and its cofactor CBP. Biochem. Biophys. Res. Commun. 228:831-7.

Janknecht, R., N. J. Wells, and T. Hunter. 1998. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev. 12:2114-9.

Jordan, J. D., K. D. Carey, P. J. Stork, and R. Iyengar. 1999. Modulation of rap activity by direct interaction of Galpha(o) with Rap1 GTPase-activating protein. J. Biol. Chem. 274:21507-10.

Jovanovic, J. N., F. Benfenati, Y. L. Siow, T. S. Sihra, J. S. Sanghera, S. L. Pelech, P. Greengard, and A. J. Czernik. 1996. Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. Proc. Natl. Acad. Sci. USA 93:3679-83.

Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S. C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld. 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 85:403-14.

Kaplan, D. R., and R. M. Stephens. 1994. Neurotrophin signal-transduction by the trk receptor. J. Neurobiol. 25:1404-1417.

Kato, S., H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, and H. K. e. al. 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270:1491-1494.

Kawasaki, H., H. Fujii, Y. Gotoh, T. Morooka, S. Shimohama, E. Nishida, and T. Hirano. 1999. Requirement for mitogen-activated protein kinase in cerebellar long term depression [In Process Citation]. J. Biol. Chem. 274:13498-502.

Kawasaki, H., G. M. Springett, N. Mochizuki, S. Toki, M. Nakaya, M. Matsuda, D. E. Housman, and A. M. Graybiel. 1998. A family of cAMP-binding proteins that directly activate Rap1. Science 282:2275-9.

Kawasaki, H., G. M. Springett, S. Toki, J. J. Canales, P. Harlan, J. P. Blumenstiel, E. J. Chen, I. A. Bany, N. Mochizuki, A. Ashbacher, M. Matsuda, D. E. Housman, and A. M. Graybiel. 1998. A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. Proc. Natl. Acad. Sci. USA 95:13278-83.

Kee, B. L., J. Arias, and M. R. Montminy. 1996. Adaptor-mediated recruitment of RNA polymerase II to a signal-dependent activator. J. Biol. Chem. 271:2373-5.

Kerkhoff, E., and U. R. Rapp. 1998. Cell cycle targets of Ras/Raf signalling. Oncogene 17:1457-62.

Khokhlatchev, A. V., B. Canagarajah, J. Wilsbacher, M. Robinson, M. Atkinson, E. Goldsmith, and M. H. Cobb. 1998. Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell 93:605-15.

Kim, J. H., and R. L. Huganir. 1999. Organization and regulation of proteins at synapses [In Process Citation]. Curr. Opin. Cell. Biol. 11:248-54.

Kim, J. H., D. Liao, L. F. Lau, and R. L. Huganir. 1998. SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. Neuron 20:683-91.

Kim, S., A. Mizoguchi, A. Kikuchi, and Y. Takai. 1990. Tissue and subcellular distributions of the smg-21/rap1/Krev-1 proteins which are partly distinct from those of c-ras p21s. Mol. Cell. Biol. 10:2645-52.

King, A. J., H. Sun, B. Diaz, D. Barnard, W. Miao, S. Bagrodia, and M. S. Marshall. 1998. The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature 396:180-3.

Kitayama, H., T. Matsuzaki, Y. Ikawa, and M. Noda. 1989. Genetic analysis of the Kirsten-*ras*-revertant 1 gene: potentiation of its tumor suppressor activity by specific point mutations. Proc. Natl. Acad. Sci. USA 87:4284-4288.

Kitayama, H., Y. Sugimoto, T. Matsuzaki, Y. Ikawa, and M. Noda. 1989. A ras-related gene with transformation suppressor activity. Cell 56:77-84.

Koizumi, S., M. D. Bootman, L. K. Bobanovic, M. J. Schell, M. J. Berridge, and P. Lipp. 1999. Characterization of elementary Ca2+ release signals in NGF-differentiated PC12 cells and hippocampal neurons. Neuron 22:125-37.

Krebs, E. G., and J. A. Beavo. 1979. Phosphorylation-dephosphorylation of enzymes. Annu. Rev. Biochem. 48:923-59.

Kretzschmar, M., J. Doody, and J. Massague. 1997. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature 389:618-22.

Kurada, P., and K. White. 1998. Ras promotes cell survival in Drosophila by downregulating hid expression [see comments]. Cell 95:319-29.

Kurino, M., K. Fukunaga, Y. Ushio, and E. Miyamoto. 1995. Activation of mitogen-activated kinase in cultured rat hippocampal neurons by stimulation of glutamate receptors. J. Neurochem. 65:1282-1289.

Kurokawa, R., D. Kalafus, M. H. Ogliastro, C. Kioussi, L. Xu, J. Torchia, M. G. Rosenfeld, and C. K. Glass. 1998. Differential use of CREB binding protein-coactivator complexes. Science 279:700-3.

Kuruvilla, R., H. Ye, and D. D. Ginty. 2000. Spatially and functionally distinct roles of the PI3-K effector pathway during NGF signaling in sympathetic neurons. Neuron 27:499-512.

Kwok, R. P., J. R. Lundblad, J. C. Chrivia, J. P. Richards, H. P. Bachinger, R. G. Brennan, S. G. Roberts, M. R. Green, and R. H. Goodman. 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 370:223-6.

Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260:315-319.

le Gallic, L., D. Sgouras, G. Beal, Jr., and G. Mavrothalassitis. 1999. Transcriptional repressor ERF is a Ras/Mitogen-activated protein kinase target that regulates cellular proliferation [In Process Citation]. Mol. Cell. Biol. 19:4121-33.

Lenormand, P., J. M. Brondello, A. Brunet, and J. Pouyssegur. 1998. Growth factor-induced p42/p44 MAPK nuclear translocation and retention requires both MAPK activation and neosynthesis of nuclear anchoring proteins. J. Cell Biol. 142:625-33.

Lev, S., H. Moreno, R. Martinez, P. Canoll, E. Peles, J. M. Musacchio, G. D. Plowman, B. Rudy, and J. Schlessinger. 1995. Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions [see comments]. Nature 376:737-45.

Lin, L. L., M. Wartmann, A. Y. Lin, J. L. Knopf, A. Seth, and R. J. Davis. 1993. cPLA2 is phosphorylated and activated by MAP kinase. Cell 72:269-278.

Lin, T. A., X. Kong, T. A. Haystead, A. Pause, G. Belsham, N. Sonenberg, and J. C. Lawrence, Jr. 1994. PHAS-I as a link between mitogen-activated protein kinase and translation initiation [see comments]. Science 266:653-6.

Liu, C., M. Peng, A. M. Laties, and R. Wen. 1998. Preconditioning with bright light evokes a protective response against light damage in the rat retina. J. Neurosci. 18:1337-44.

Liu, Y. Z., J. C. Chrivia, and D. S. Latchman. 1998. Nerve growth factor up-regulates the transcriptional activity of CBP through activation of the p42/p44(MAPK) cascade. J. Biol. Chem. 273:32400-7.

Liu, Y. Z., N. S. Thomas, and D. S. Latchman. 1999. CBP associates with the p42/p44 MAPK enzymes and is phosphorylated following NGF treatment. Neuroreport 10:1239-43.

Lohof, A. M., M. Quillan, Y. Dan, and M. M. Poo. 1992. Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. J. Neurosci. 12:1253-61.

Lowy, D. R., and B. M. Willumsen. 1993. Function and regulation of ras. Annu. Rev. Biochem. 62:851-91.

Lu, N., and E. DiCicco-Bloom. 1997. Pituitary adenylate cyclase-activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells. Proc. Natl. Acad. Sci. USA 94:3357-62.

Ludwig, S., K. Engel, A. Hoffmeyer, G. Sithanandam, B. Neufeld, D. Palm, M. Gaestel, and U. R. Rapp. 1996. 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. Mol. Cell. Biol. 16:6687-97.

Malenka, R. C. 1994. Synaptic plasticity in the hippocampus: LTP and LTD. Cell 78:535-8.

Mao, Z., A. Bonni, F. Xia, M. Nadal-Vicens, and M. E. Greenberg. 1999. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science 286:785-90.

Marais, R., Y. Light, H. F. Paterson, and C. J. Marshall. 1995. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J. 14:3136-3145.

Mark, M. D., Y. Liu, S. T. Wong, T. R. Hinds, and D. R. Storm. 1995. Stimulation of neurite outgrowth in PC12 cells by EGF and KCl depolarization: a Ca(2+)-independent phenomenon. J. Cell Biol. 130:701-10.

Mark, M. D., and D. R. Storm. 1997. Coupling of epidermal growth factor (EGF) with the antiproliferative activity of cAMP induces neuronal differentiation. J. Biol. Chem. 272:17238-44.

Mark, M. D., and D. R. Storm. 1997. Coupling of Epidermal Growth Factor (EGF) with the Antiproliferative Actvity of cAMP Induces Neuronal Differentiation. J Biol. Chem. 272:17238-17244.

Marshall, C. J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-85.

Martin, K. C., and E. R. Kandel. 1996. Cell adhesion molecules, CREB, and the formation of new synaptic connections. Neuron 17:567-70.

Martin, K. C., D. Michael, J. C. Rose, M. Barad, A. Casadio, H. Zhu, and E. R. Kandel. 1997. MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in Aplysia. Neuron 18:899-912.

Maruta, H., and A. W. Burgess. 1994. Regulation of the Ras signalling network. Bioessays 16:489-96.

Mason, C. S., C. J. Springer, R. G. Cooper, G. Superti-Furga, C. J. Marshall, and R. Marais. 1999. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. EMBO J. 18:2137-48.

Matthews, G. 1996. Neurotransmitter release. Annu. Rev. Neurosci. 19:219-33.

Matthews, R. P., C. R. Guthrie, L. M. Wailes, X. Zhao, A. R. Means, and G. S. McKnight. 1994. Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol. Cell. Biol. 14:6107-16.

McCarthy, S. A., D. Chen, B.-S. Yang, J. J. g. R. H. Cherwinski, S.-R. Chen, M. Klagsbrun, C. A. Hauser, M. C. Ostrowski, and M. McMahon. 1997. rapid

phosphorylation of Ets-2 accompanies mitogen-activated protein kinase activation and the induction of heparin-binding epidermal growth factor gene expression by oncogenic Raf-1. Mol. Cell. Biol. 17:2401-2412.

Meakin, S. O., J. I. MacDonald, E. A. Gryz, C. J. Kubu, and J. M. Verdi. 1999. The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. J. Biol. Chem. 274:9861-70.

Meyer-Franke, A., M. R. Kaplan, F. W. Pfrieger, and B. A. Barres. 1995. Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron 15:805-819.

Meyer-Franke, A., G. A. Wilkinson, A. Kruttgen, M. Hu, E. Munro, M. G. Hanson, Jr., L. F. Reichardt, and B. A. Barres. 1998. Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron 21:681-93.

Mikkola, I., J. A. Bruun, G. Bjorkoy, T. Holm, and T. Johansen. 1999. Phosphorylation of the transactivation domain of pax6 by extracellular signal-regulated kinase and p38 mitogen-activated protein kinase [In Process Citation]. J. Biol. Chem. 274:15115-26.

Ming, G. L., H. J. Song, B. Berninger, C. E. Holt, M. Tessier-Lavigne, and M. M. Poo. 1997. cAMP-dependent growth cone guidance by netrin-1. Neuron 19:1225-35.

Miranti, C. K., D. D. Ginty, G. Huang, T. Chatila, and M. E. Greenberg. 1995. Calcium activates serum response factor-dependent transcription by a Ras- and Elk-1-independent mechanism that involves a Ca2+/calmodulin- dependent kinase. Mol. Cell. Biol. 15:3672-84.

Misra, R. P., A. Bonni, C. K. Miranti, V. M. Rivera, M. Sheng, and M. E. Greenberg. 1994. L-type voltage-sensitive calcium channel activation stimulates gene expression by a serum response factor-dependent pathway. J. Biol. Chem. 269:25483-25493.

Montminy, M. 1997. Transcriptional regulation by cyclic AMP. Annu. Rev. Biochem. 66:807-22.

Montminy, M., P. Brindle, J. Arias, K. Ferreri, and R. Armstrong. 1996. Regulation of somatostatin gene transcription by cAMP. Adv Pharmacol 36:1-13.

Montminy, M. R., and L. M. Bilezikjian. 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328:175-8.

Montminy, M. R., G. A. Gonzalez, and K. K. Yamamoto. 1990. Regulation of cAMP-inducible genes by CREB. Trends Neurosci. 13:184-8.

Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA 83:6682-6686.

Moon, C., Y. K. Sung, R. Reddy, and G. V. Ronnett. 1999. Odorants induce the phosphorylation of the cAMP response element binding protein in olfactory receptor neurons. Proc. Natl. Acad. Sci. USA 96:14605-10.

Moore, A. N., M. N. Waxham, and P. K. Dash. 1996. Neuronal activity increases the phosphorylation of the transcription factor cAMP response element-binding protein (CREB) in rat hippocampus and cortex. J. Biol. Chem. 271:14214-20.

Morgan, J. I., and T. Curran. 1986. Role of ion flux in the control of c-fos expression. Nature 322:552-5.

Morgan, J. I., and T. Curran. 1989. Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. Trends Neurosci. 12:459-62.

Morrison, D. K., and R. E. Cutler. 1997. The complexity of Raf-1 regulation. Curr. Opin. Cell. Biol. 9:174-9.

Mukherjee, P. K., M. A. DeCoster, F. Z. Campbell, R. J. Davis, and N. G. Bazan. 1999. Glutamate receptor signaling interplay modulates stress-sensitive mitogen-activated protein kinases and neuronal cell death. J. Biol. Chem. 274:6493-8.

Murphy, T. H., L. A. Blatter, R. V. Bhat, R. S. Fiore, W. G. Wier, and J. M. Baraban. 1994. Differential regulation of calcium/calmodulin-dependent protein kinase II and p42 MAP kinase activity by synaptic transmission. J. Neurosci. 14:1320-31.

Murray, B., A. Alessandrini, A. J. Cole, A. G. Yee, and E. J. Furshpan. 1998. Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. Proc. Natl. Acad. Sci. USA 95:11975-80.

Muzzio, I. A., A. Morozov, D. G. Winder, and E. R. Kandel. 2000. The cAMP-activated small GTPase Rap1 provides dual regulation of the MAP kinase cascade and is critical for plasticity in the hippocampal area CA1. Soc. Neurosci. Abstr. 26:133.3.

Nakajima, T., A. Fukamizu, J. Takahashi, F. H. Gage, T. Fisher, J. Blenis, and M. R. Montminy. 1996. The signal-dependent coactivator CBP is a nuclear target for pp90RSK. Cell 86:465-74.

Nakajima, T., C. Uchida, S. F. Anderson, C. G. Lee, J. Hurwitz, J. D. Parvin, and M. Montminy. 1997. RNA helicase A mediates association of CBP with RNA polymerase II. Cell 90:1107-12.

Nayak, A., D. J. Zastrow, R. Lickteig, N. R. Zahniser, and M. D. Browning. 1998. Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis. Nature 394:680-3.

Nguyen, P. V., and E. R. Kandel. 1996. A macromolecular synthesis-dependent late phase of long-term potentiation requiring cAMP in the medial perforant pathway of rat hippocampal slices. J. Neurosci. 16:3189-3198.

Nicoll, R. A., and R. C. Malenka. 1995. Contrasting properties of two forms of long-term potentiation in the hippocampus. Nature 377:115-118.

O'Connor, V., G. J. Augustine, and H. Betz. 1994. Synaptic vesicle exocytosis: molecules and models. Cell 76:785-7.

Obrietan, K., S. Impey, D. Smith, J. Athos, and D. R. Storm. 1999. Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. J. Biol. Chem. 274:17748-56.

Obrietan, K., S. Impey, and D. R. Storm. 1998. Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. Nat. Neurosci. 1:693-700.

Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87:953-9.

Ohmitsu, M., K. Fukunaga, H. Yamamoto, and E. Miyamoto. 1999. Phosphorylation of myristoylated alanine-rich protein kinase C substrate by mitogen-activated protein kinase in cultured rat hippocampal neurons following stimulation of glutamate receptors. J. Biol. Chem. 274:408-17.

Parker, D., K. Ferreri, T. Nakajima, V. J. LaMorte, R. Evans, S. C. Koerber, C. Hoeger, and M. R. Montminy. 1996. Phosphorylation of CREB at Ser-133 induces complex formation with CREB- binding protein via a direct mechanism. Mol. Cell. Biol. 16:694-703.

Parker, D., U. S. Jhala, I. Radhakrishnan, M. B. Yaffe, C. Reyes, A. I. Shulman, L. C. Cantley, P. E. Wright, and M. Montminy. 1998. Analysis of an activator:coactivator complex reveals an essential role for secondary structure in transcriptional activation. Mol Cell 2:353-9.

Pawson, T. 1995. Protein modules and signalling networks. Nature 373:573-579.

Pawson, T., and J. D. Scott. 1997. Signaling through scaffold, anchoring, and adaptor proteins. Science 278:2075-80.

Peers, B., P. Monget, M. A. Nalda, M. L. Voz, M. Berwaer, A. Belayew, and J. A. Martial. 1991. Transcriptional induction of the human prolactin gene by cAMP requires two cis-acting elements and at least the pituitary-specific factor Pit-1. J. Biol. Chem. 266:18127-34.

Peers, B., M. L. Voz, P. Monget, M. Mathy-Hartert, M. Berwaer, A. Belayew, and J. A. Martial. 1990. Regulatory elements controlling pituitary-specific expression of the human prolactin gene. Mol. Cell. Biol. 10:4690-700.

Peter, M., J. S. Sanghera, S. L. Pelech, and E. A. Nigg. 1992. Mitogen-activated protein kinases phosphorylate nuclear lamins and display sequence specificity overlapping that of mitotic protein kinase p34cdc2. Eur J Biochem 205:287-94.

Pham, N., I. Cheglakov, C. A. Koch, C. L. de Hoog, M. F. Moran, and D. Rotin. 2000. The guanine nucleotide exchange factor CNrasGEF activates ras in response to cAMP and cGMP. Curr. Biol. 10:555-8.

Pierrat, B., J. S. Correia, J. L. Mary, M. Tomas-Zuber, and W. Lesslauer. 1998. RSK-B, a novel ribosomal S6 kinase family member, is a CREB kinase under dominant control of p38alpha mitogen-activated protein kinase (p38alphaMAPK). J. Biol. Chem. 273:29661-71.

Pircher, T. J., H. Petersen, J. A. Gustafsson, and L. A. Haldosen. 1999. Extracellular signal-regulated kinase (ERK) interacts with signal transducer and activator of transcription (STAT) 5a. Mol. Endocrinol. 13:555-65.

Price, M. A., A. E. Rogers, and R. Treisman. 1995. Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). EMBO J. 14:2589-601.

Qian, X., A. Riccio, Y. Zhang, and D. D. Ginty. 1998. Identification and characterization of novel substrates of Trk receptors in developing neurons. Neuron 21:1017-29.

Qiu, W., S. Zhuang, F. C. von Lintig, G. R. Boss, and R. B. Pilz. 2000. Cell type-specific regulation of B-Raf kinase by cAMP and 14-3-3 proteins. J. Biol. Chem. 275:31921-9.

Rebay, I., and G. M. Rubin. 1995. Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell 81:857-66.

Riccio, A., S. Ahn, C. M. Davenport, J. A. Blendy, and D. D. Ginty. 1999. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286:2358-61.

Riccio, A., B. A. Pierchala, C. L. Ciarallo, and D. D. Ginty. 1997. An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons [see comments]. Science 277:1097-100.

Roberson, E. D., J. D. English, J. P. Adams, J. C. Selcher, C. Kondratick, and J. D. Sweatt. 1999. The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J. Neurosci. 19:4337-48.

Robinson, M. J., S. A. Stippec, E. Goldsmith, M. A. White, and M. H. Cobb. 1998. A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation. Curr. Biol. 8:1141-50.

Rodriguez-Viciana, P., P. H. Warne, R. Dhand, B. Vanhaesebroeck, I. Gout, M. J. Fry, M. D. Waterfield, and J. Downward. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527-32.

Roesler, W. J. 2000. What is a cAMP response unit? Mol Cell Endocrinol 162:1-7.

Roesler, W. J., J. Simard, J. G. Graham, and P. J. McFie. 1994. Characterization of the liver-specific component of the cAMP response unit in the phosphoenolpyruvate carboxykinase (GTP) gene promoter. J. Biol. Chem. 269:14276-83.

Rommel, C., B. A. Clarke, S. Zimmermann, L. Nunez, R. Rossman, K. Reid, K. Moelling, G. D. Yancopoulos, and D. J. Glass. 1999. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. Science 286:1738-41.

Rosen, L. B., D. D. Ginty, M. J. Weber, and M. E. Greenberg. 1994. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 12:1207-21.

Rosen, L. B., and M. E. Greenberg. 1996. Stimulation of growth factor receptor signal transduction by activation of voltage-sensitive calcium channels. Proc. Natl. Acad. Sci. USA 93:1113-1118.

Rosenmund, C., D. W. Carr, S. E. Bergson, G. Nilaver, J. D. Scott, and G. L. Westbrook. 1994. Anchoring of protein kinase A is required for modulation of AMPA/kinase receptors on hippocampal neurons. Nature 368:853-856.

Rubinfeld, B., S. Munemitsu, R. Clark, L. Conroy, K. Watt, W. J. Crosier, F. McCormick, and P. Polakis. 1991. Molecular cloning of a GTPase activating protein specific for the Krev-1 protein p21rap1. Cell 65:1033-42.

Runden, E., P. O. Seglen, F. M. Haug, O. P. Ottersen, T. Wieloch, M. Shamloo, and J. H. Laake. 1998. Regional selective neuronal degeneration after protein phosphatase inhibition in hippocampal slice cultures: evidence for a MAP kinase-dependent mechanism. J. Neurosci. 18:7296-305.

Rusanescu, G., H. Qi, S. M. Thomas, J. S. Brugge, and S. Halegoua. 1995. Calcium influx induces neurite growth through a Src-Ras signaling cassette. Neuron 15:1415-25.

Rydel, R. E., and L. A. Greene. 1988. cAMP analogs promote survival and neurite outgrowth in cultures of rat sympathetic and sensory neurons independently of nerve growth factor. Proc. Natl. Acad. Sci. USA 85:1257-1261.

Sah, P., and E. M. McLachlan. 1991. Ca(2+)-activated K+ currents underlying the afterhyperpolarization in guinea pig vagal neurons: a role for Ca(2+)-activated Ca2+ release. Neuron 7:257-64.

Sassone-Corsi, P., J. Visvader, L. Ferland, P. L. Mellon, and I. M. Verma. 1988. Induction of proto-oncogene fos transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. Genes Dev. 2:1529-38.

Schaeffer, H. J., A. D. Catling, S. T. Eblen, L. S. Collier, A. Krauss, and M. J. Weber. 1998. MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade [see comments]. Science 281:1668-71.

Schaeffer, H. J., and M. J. Weber. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol. Cell. Biol. 19:2435-44.

Schlessinger, J. 1993. How receptor tyrosine kinases activate Ras. Trends Biochem. Sci. 18:273-5.

Schlessinger, J. 1994. SH2/SH3 signaling proteins. Curr Opin Genet Dev 4:25-30.

Schlessinger, J., and D. Bar-Sagi. 1994. Activation of Ras and other signaling pathways by receptor tyrosine kinases. Cold Spring Harb Symp Quant Biol 59:173-9.

Schulz, S., and V. Hollt. 1998. Opioid withdrawal activates MAP kinase in locus coeruleus neurons in morphine-dependent rats in vivo. Eur. J. Neurosci. 10:1196-201.

Segal, R., and M. Greenberg. 1996. Intracellular signaling pathways axtivated by neurotrophic factors. Annu. Rev. Neurosci. 19:463-489.

Selfors, L. M., and M. J. Stern. 1994. MAP kinase function in C. elegans. Bioessays 16:301-4.

Senger, D. L., and R. B. Campenot. 1997. Rapid retrograde tyrosine phosphorylation of trkA and other proteins in rat sympathetic neurons in compartmented cultures. J. Cell Biol. 138:411-21.

Sgambato, V., C. Pages, M. Rogard, M. J. Besson, and J. Caboche. 1998. Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. J. Neurosci. 18:8814-25.

Sgambato, V., P. Vanhoutte, C. Pages, M. Rogard, R. Hipskind, M. J. Besson, and J. Caboche. 1998. In vivo expression and regulation of Elk-1, a target of the

extracellular-regulated kinase signaling pathway, in the adult rat brain. J. Neurosci. 18:214-26.

Shaywitz, A. J., and M. E. Greenberg. 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu. Rev. Biochem. 68:821-861.

Sheng, H. Z., R. D. Fields, and P. G. Nelson. 1993. Specific regulation of immediate early genes by patterned neuronal activity. J. Neurosci. Res. 35:459-67.

Sheng, M., S. T. Dougan, G. McFadden, and M. E. Greenberg. 1988. Calcium and growth factor pathways of c-fos transcriptional activation require distinct upstream regulatory sequences. Mol. Cell. Biol. 8:2787-96.

Sheng, M., G. McFadden, and M. E. Greenberg. 1990. Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron 4:571-582.

Sheng, M., M. A. Thompson, and M. E. Greenberg. 1991. CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252:1427-1430.

Shieh, P. B., S.-C. Hu, K. Bobb, T. Timmusk, and A. Ghosh. 1998. Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20:727-740.

Silva, A. J., J. H. Kogan, P. W. Frankland, and S. Kida. 1998. CREB and memory. Annu. Rev. Neurosci. 21:127-48.

Simonds, W. F. 1999. G protein regulation of adenylate cyclase. Trends Pharm. 20:66-73.

Smith, J. A., C. E. Poteet-Smith, K. Malarkey, and T. W. Sturgill. 1999. Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo. J. Biol. Chem. 274:2893-8.

Soderling, S. H., and J. A. Beavo. 2000. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. Curr. Opin. Cell. Biol. 12:174-9.

Song, H. J., G. L. Ming, and M. M. Poo. 1997. cAMP-induced switching in turning direction of nerve growth cones. Nature 388:275-9.

Stokoe, D., D. G. Campbell, S. Nakielny, H. Hidaka, S. J. Leevers, C. Marshall, and P. Cohen. 1992. MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. EMBO J. 11:3985-94.

Sun, P., H. Enslen, P. S. Myung, and R. A. Maurer. 1994. Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev. 8:2527-2539.

Sundaram, M., and M. Han. 1996. Control and integration of cell signaling pathways during C. elegans vulval development. Bioessays 18:473-80.

Swank, M. W. 2000. Phosphorylation of MAP kinase and CREB in mouse cortex and amygdala during taste aversion learning. Neuroreport 11:1625-30.

Sweatt, J. D. 2001. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J. Neurochem. 76:1-10.

Swope, D. L., C. L. Mueller, and J. C. Chrivia. 1996. CREB-binding protein activates transcription through multiple domains. J. Biol. Chem. 271:28138-45.

Takishima, K., I. Griswold-Prenner, T. Ingebritsen, and M. R. Rosner. 1991. Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3-L1 cells is an EGF-stimulated "MAP" kinase. Proc. Natl. Acad. Sci. USA 88:2520-4.

Tao, X., S. Finkbeiner, D. B. Arnold, A. J. Shaywitz, and M. E. Greenberg. 1998. Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism [published erratum appears in Neuron 1998 Jun;20(6):1297]. Neuron 20:709-26.

Taubenfeld, S. M., K. A. Wiig, B. Monti, B. Dolan, G. Pollonini, and C. M. Alberini. 2001. Fornix-Dependent Induction of Hippocampal CCAAT Enhancer-Binding Protein {beta} and {delta} Co-Localizes with Phosphorylated cAMP

Response Element-Binding Protein and Accompanies Long-Term Memory Consolidation. J. Neurosci. 21:84-91.

Taylor, S. S., J. A. Buechler, and W. Yonemoto. 1990. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu. Rev. Biochem. 59:971-1005.

Thomas, K. L., S. Laroche, M. L. Errington, T. V. Bliss, and S. P. Hunt. 1994. Spatial and temporal changes in signal transduction pathways during LTP. Neuron 13:737-45.

Thompson, M. A., D. D. Ginty, A. Bonni, and M. E. Greenberg. 1995. L-type voltage-sensitive Ca2+ channel activation regulates c-fos transcription at multiple levels. J. Biol. Chem. 270:4224-35.

Tzivion, G., Z. Luo, and J. Avruch. 1998. A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. Nature 394:88-92.

Vaillant, A. R., I. Mazzoni, C. Tudan, M. Boudreau, D. R. Kaplan, and F. D. Miller. 1999. Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J. Cell Biol. 146:955-66.

van den Berghe, N., R. H. Cool, G. Horn, and A. Wittinghofer. 1997. Biochemical characterization of C3G: an exchange factor that discriminates between Rap1 and Rap2 and is not inhibited by Rap1A(S17N). Oncogene 15:845-50.

van der Geer, P., T. Hunter, and R. A. Lindberg. 1994. Receptor protein-tyrosine kinases and their signal transduction pathways. Annu. Rev. Cell Biol. 10:251-337.

Vanhoutte, P., J. V. Barnier, B. Guibert, C. Pages, M. J. Besson, R. A. Hipskind, and J. Caboche. 1999. Glutamate induces phosphorylation of elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices [In Process Citation]. Mol. Cell. Biol. 19:136-46.

Veeranna, N. D. Amin, N. G. Ahn, H. Jaffe, C. A. Winters, P. Grant, and H. C. Pant. 1998. Mitogen-activated protein kinases (Erk1,2) phosphorylate Lys-Ser-Pro (KSP) repeats in neurofilament proteins NF-H and NF-M. J. Neurosci. 18:4008-21.

Villalba, M., J. Bockaert, and L. Journot. 1997. Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen- activated protein kinase (MAP kinase) pathway. J. Neurosci. 17:83-90.

Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 74:205-14.

Vossler, M. R., H. Yao, R. D. York, M. G. Pan, C. S. Rim, and P. J. Stork. 1997. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 89:73-82.

Walton, M. R., and I. Dragunow. 2000. Is CREB a key to neuronal survival? Trends Neurosci. 23:48-53.

Waskiewicz, A. J., J. C. Johnson, B. Penn, M. Mahalingam, S. R. Kimball, and J. A. Cooper. 1999. Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. Mol. Cell. Biol. 19:1871-80.

Westphal, R. S., S. J. Tavalin, J. W. Lin, N. M. Alto, I. D. Fraser, L. K. Langeberg, M. Sheng, and J. D. Scott. 1999. Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. Science 285:93-6.

Whitfield, J. F., A. L. Boynton, J. P. MacManus, M. Sikorska, and B. K. Tsang. 1979. The regulation of cell proliferation by calcium and cyclic AMP. Mol Cell Biochem 27:155-79.

Whitmarsh, A. J., J. Cavanagh, C. Tournier, J. Yasuda, and R. J. Davis. 1998. A mammalian scaffold complex that selectively mediates MAP kinase activation [see comments]. Science 281:1671-4.

Wickman, K., and D. E. Clapham. 1995. Ion channel regulation by G proteins. Physiol. Rev. 75:865-85.

Winder, D. G., K. C. Martin, I. A. Muzzio, D. Rohrer, A. Chruscinski, B. Kobilka, and E. R. Kandel. 1999. ERK plays a regulatory role in induction of LTP by theta frequency stimulation and its modulation by beta-adrenergic receptors. Neuron 24:715-26.

Wong, S. T., J. Athos, X. A. Figueroa, V. V. Pineda, M. L. Schaefer, C. C. Chavkin, L. J. Muglia, and D. R. Storm. 1999. Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. Neuron 23:787-98.

Wu, J., P. Dent, T. Jelinek, A. Wolfman, M. J. Weber, and T. W. Sturgill. 1993. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3', 5'-monophosphate. Science 262:1065-1068.

Xia, Z., H. Dudek, C. K. Miranti, and M. E. Greenberg. 1996. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J. Neurosci. 16:5425-36.

Xia, Z., and D. R. Storm. 1997. Calmodulin-regulated adenylyl cyclases and neuromodulation. Curr. Opin. Neurobiol. 7:391-396.

Xia, Z. G., C. D. Refsdal, K. M. Merchant, D. M. Dorsa, and D. R. Storm. 1991. Distribution of mRNA for the calcodulin-sensitive adenylate cyclase in rat brain: expresssion in areas associated with learning and memory. Neuron 6:431-443.

Xing, J., D. D. Ginty, and M. E. Greenberg. 1996. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 273:959-63.

Xing, L., V. K. Gopal, and P. G. Quinn. 1995. cAMP response element-binding protein (CREB) interacts with transcription factors IIB and IID. J. Biol. Chem. 270:17488-93.

Xu, L., R. M. Lavinsky, J. S. Dasen, S. E. Flynn, E. M. McInerney, T. M. Mullen, T. Heinzel, D. Szeto, E. Korzus, R. Kurokawa, A. K. Aggarwal, D. W. Rose, C. K. Glass, and M. G. Rosenfeld. 1998. Signal-specific co-activator domain requirements for Pit-1 activation. Nature 395:301-6.

Yamamoto, K. K., G. A. Gonzalez, W. H. d. Biggs, and M. R. Montminy. 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334:494-8.

Yao, H., K. Labudda, C. Rim, P. Capodieci, M. Loda, and P. J. S. Stork. 1995. Cyclic adenosine monophosphate can convert epidermal growth factor into a differentiating factor in neuronal cells. J. Biol. Chem. 270:20748-20753.

Yao, H., R. D. York, A. Misra-Press, D. W. Carr, and P. J. Stork. 1998. The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated kinases and gene expression by nerve growth factor. J. Biol. Chem. 273:8240-7.

York, R. D., D. C. Molliver, S. S. Grewal, P. E. Stenberg, E. W. McCleskey, and P. J. Stork. 2000. Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via ras and rap1. Mol. Cell. Biol. 20:8069-83.

York, R. D., H. Yao, T. Dillon, C. L. Ellig, S. P. Eckert, E. W. McCleskey, and P. J. Stork. 1998. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392:622-6.

Yukawa, K., T. Tanaka, S. Tsuji, and S. Akira. 1998. Expressions of CCAAT/Enhancer-binding proteins beta and delta and their activities are intensified by cAMP signaling as well as Ca2+/calmodulin kinases activation in hippocampal neurons. J. Biol. Chem. 273:31345-51.

Yun, H. Y., M. Gonzalez-Zulueta, V. L. Dawson, and T. M. Dawson. 1998. Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of p21ras. Proc. Natl. Acad. Sci. USA 95:5773-8.

Zagotta, W. N., and S. A. Siegelbaum. 1996. Structure and function of cyclic nucleotide-gated channels. Annu. Rev. Neurosci. 19:235-63.

Zanassi, P., M. Paolillo, A. Feliciello, E. V. Avvedimento, V. Gallo, and S. Schinelli. 2001. Cyclic AMP-dependent protein kinase induces CREB phosphorylation via an intracellular calcium release/ERK-dependent pathway in striatal neurons. J. Biol. Chem. 3:3.

Zanger, K., L. E. Cohen, K. Hashimoto, S. Radovick, and F. E. Wondisford. 1999. A novel mechanism for cyclic adenosine 3',5'-monophosphate regulation of gene expression by CREB-binding protein. Mol. Endocrinol. 13:268-75.

Zheng, C. F., and K. L. Guan. 1993. Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. J. Biol. Chem. 268:11435-9.

Zheng, C. F., and K. L. Guan. 1993. Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. J. Biol. Chem. 268:23933-9.

Zheng, J. Q., Z. Zheng, and M. Poo. 1994. Long-range signaling in growing neurons after local elevation of cyclic AMP-dependent activity. J. Cell Biol. 127:1693-701.

Zhou, L., E. M. Kasperek, and B. J. Nicholson. 1999. Dissection of the molecular basis of pp60(v-src) induced gating of connexin 43 gap junction channels [In Process Citation]. J. Cell Biol. 144:1033-45.

Zimmermann, S., and K. Moelling. 1999. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science 286:1741-4.

Zucker, R. S. 1999. Calcium- and activity-dependent synaptic plasticity. Curr. Opin. Neurobiol. 9:305-13.

Zwartkruis, F. J., R. M. Wolthuis, N. M. Nabben, B. Franke, and J. L. Bos. 1998. Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. EMBO J. 17:5905-12.

Zwick, E., H. Daub, N. Aoki, Y. Yamaguchi-Aoki, I. Tinhofer, K. Maly, and A. Ullrich. 1997. Critical role of calcium-dependent epidermal growth factor receptor transactivation in PC12 cell membrane depolarization and bradykinin signaling. J. Biol. Chem. 272:24767-24770.