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MAPK regulation of gene expression in the central nervous system

J. Paige Adams, Erik D. Roberson, Joey D. English, Joel C. Selcher and **J. David Sweatt**[1]

Division of Neuroscience, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas 77030, USA, Email: jsweatt@bcm.tmc.edu

Abstract. Long-term potentiation (LTP), a cellular model for long-term memory, is generally acknowledged to consist of both a short-term phase that is characterized by a dependence on autonomous protein kinase activity, and a long-term phase that is characterized by a dependence on changes in gene expression and new protein synthesis. Similarly, long-term memory exhibits a dependence on gene expression and altered protein synthesis. Recent evidence indicates that the mitogen-activated protein kinase (MAPK) cascade plays a role in both LTP and long-term memory. The MAPK cascade has heretofore largely been studied in the context of cell division and proliferation and as such, mechanisms for the regulation of gene expression by the MAPK cascade have received considerable attention. Given the possible role of altered gene expression in the late phase of LTP and in long-term memory, we evaluated the capacity of the MAPK ERK (extracellular signal-regulated kinase) to regulate phosphorylation of the transcription factor cAMP response element binding protein (CREB) in hippocampal area CA1. Our studies indicate a critical role for the MAPK cascade in the regulation of CREB phosphorylation in the hippocampus.

[1]To whom correspondence should be addressed

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INTRODUCTION

LTP consists of two phases, an early phase and a late phase. The late phase, which typically lasts longer than two hours and up to 7 h using *in vitro* techniques, is dependent on protein synthesis. Thus, application of protein synthesis inhibitors blocks the late phase of LTP in the hippocampus (Frey et al. 1988). The machinery involved in new mRNA and protein synthesis includes the activation of transcription factors, which bind to consensus elements on DNA and thus initiate transcription. One such transcription factor that has been shown to be involved in hippocampal LTP is CREB. Not only has CREB been shown to be involved in this form of synaptic plasticity, but evidence exists implicating CREB in hippocampus-dependent long-term memory.

The regulation of CREB in hippocampal neurons has been shown to involve several protein kinases, including the cAMP-dependent protein kinase, PKA. We present evidence here that one effect of the activation of PKA in the hippocampus is the activation of the MAPK ERK. Interestingly, ERK has been shown to be required for the induction of the late-phase of LTP in the hippocampus; in addition, ERK has been implicated in several forms of hippocampus-dependent long-term memory. We therefore hypothesized that PKA regulation of CREB might involve the MAPK cascade. We found that activation of the PKA cascade led to an increase in the phosphorylation of CREB; importantly, we show here that the increase occurs mainly through the intervening kinase ERK. Similarly, we found that activation of PKC led to an increase in the phosphorylation of CREB; this increase seems to act at least in part through the intervening kinase ERK as well. Thus, it seems that the regulation of CREB in hippocampal synaptic plasticity may involve the activation of the PKA cascade and/or PKC, as well as the activation of the MAPK cascade.

MAPK

In the last decade, a new class of signaling cascades has been dissected: the MAPK cascades. They are distinguished by a characteristic core cascade of three kinases (Cobb and Goldsmith 1995, Marshall 1995, Fig.1). The first is a so-called MAP kinase kinase kinase (MAPKKK) which activates the second, a MAP kinase kinase (MAPKK), by serine/threonine phosphorylation. MAPKKs are dual specificity kinases that in turn activate a MAP kinase by phosphorylating threonine and tyrosine moieties.

MAP kinase was originally identified as an insulin-stimulated kinase that phosphorylates microtubule associated protein 2 (MAP2) *in vitro*, and thus was initially called MAP2 kinase (Ray and Sturgill 1988). Subsequent studies revealed that many different mitogenic stimuli could elicit MAP2 kinase activation, and this kinase was renamed the mitogen-activated protein kinase (MAPK, Rossomando et al. 1989). MAPK was later cloned and provided with yet another name: the extracellular signal-regulated protein kinase (ERK, Boulton et al. 1991). To date, three isoforms have been identified, each from a distinct gene: a 44 kD isoform, p44 MAPK (ERK1); a 42 kD isoform, p42 MAPK (ERK2); and a 62 kD isoform, p62 MAPK (ERK3). p44 MAPK and p42 MAPK share 90% amino acid identity in the catalytic domain and are thought to be functionally redundant; p62 MAPK, however, shares only 50% identity with p44 MAPK/p42 MAPK and appears to be functionally distinct (Boulton et al. 1991, Seger and Krebs 1995).

Regulation of MAPK activation

The best characterized pathway leading to MAPK activation is that initiated by growth factors such as epidermal growth factor (EGF) and nerve growth factor (NGF) (Davis 1993, Seger and Krebs 1995, Downward 1996, Marais and Marshall 1996). Binding of a growth factor to its receptor leads to the activation of the protein tyrosine kinase (PTK) catalytic domain found in the cytoplasmic region of the transmembrane receptor. While this domain may phosphorylate other protein substrates, at present it is thought that the main consequence of activation is autophosphorylation of the receptor at specific tyrosine residues. This leads to the recruitment of adapter proteins such as GRB2, which bind selectively to phosphotyrosine residues *via* SH2 (src homology 2) domains. GRB2 then binds and activates mSOS, a guanine nucleotide exchange protein. mSOS in turn promotes GDP to GTP exchange by the small, membrane--associated G protein, Ras. Activated Ras then recruits the serine/threonine protein kinase Raf to the membrane, leading to its activation. Finally, Raf phosphorylates and activates the dual-specific protein kinase MEK (MAPK/ERK kinase, also known as MAP kinase kinase), which in turn activates MAPK *via* phosphorylations on a single threonine and single tyrosine resi-

due. This initial insight that MAPKs must be phosphorylated on both threonine and tyrosine residues in order to be active was made by Sturgill and colleagues (Anderson et al. 1990). From this observation, it was proposed that MAPK activation required the convergence of two independent protein kinase pathways: a protein serine/threonine kinase and a protein tyrosine kinase. It is now clear, however, that the single kinase MEK mediates these phosphorylation events (Crews et al. 1992, Crews and Erikson 1992, Zheng and Guan 1993).

MAPKs can also be activated independently of growth factor receptor stimulation. Cytoplasmic PTKs such as src kinases and PYK2 are strong activators of MAPKs in many cell types (Cantley et al. 1991, Alessandrini et al. 1992, Gardner et al. 1993, Gause et al. 1993, Lev et al. 1995). In addition, certain G protein-coupled serpentine receptors can trigger MAPK activation, including TxA_2/PGH_2, PGF_2 , -thrombin, angiotensin II, $_1$-adrenergic, $_2$-adrenergic, muscarinic, D_2 dopamine and A1 adenosine receptors (van Biesen et al. 1996, Della Rocca et al. 1997, Gutkind 1998). Though the mechanism of activation by G protein-coupled receptors is poorly understood, it is proposed that such activation proceeds *via* stimulation of either Ras or Raf. In addition, AMPA receptor stimulation has been shown to lead to increased ERK activation in primary rat cortical cell cultures; this activation was shown to require Ca^{2+} influx and involve G-protein subunits and Ras (Wang and Durkin 1995). Overall therefore, while many diverse stimuli can elicit MAPK activation, all are thought to do so by converging upon a signaling module consisting of the three protein kinases: a MAP kinase kinase kinase (Raf), MAP kinase kinase (MEK) and MAP kinase itself (ERK).

A superfamily of MAPK signaling cascades

The elucidation of this biochemical pathway was followed by discoveries of a superfamily of several signaling cascades that utilize this three-kinase signaling module; each pathway is now considered to be a distinct

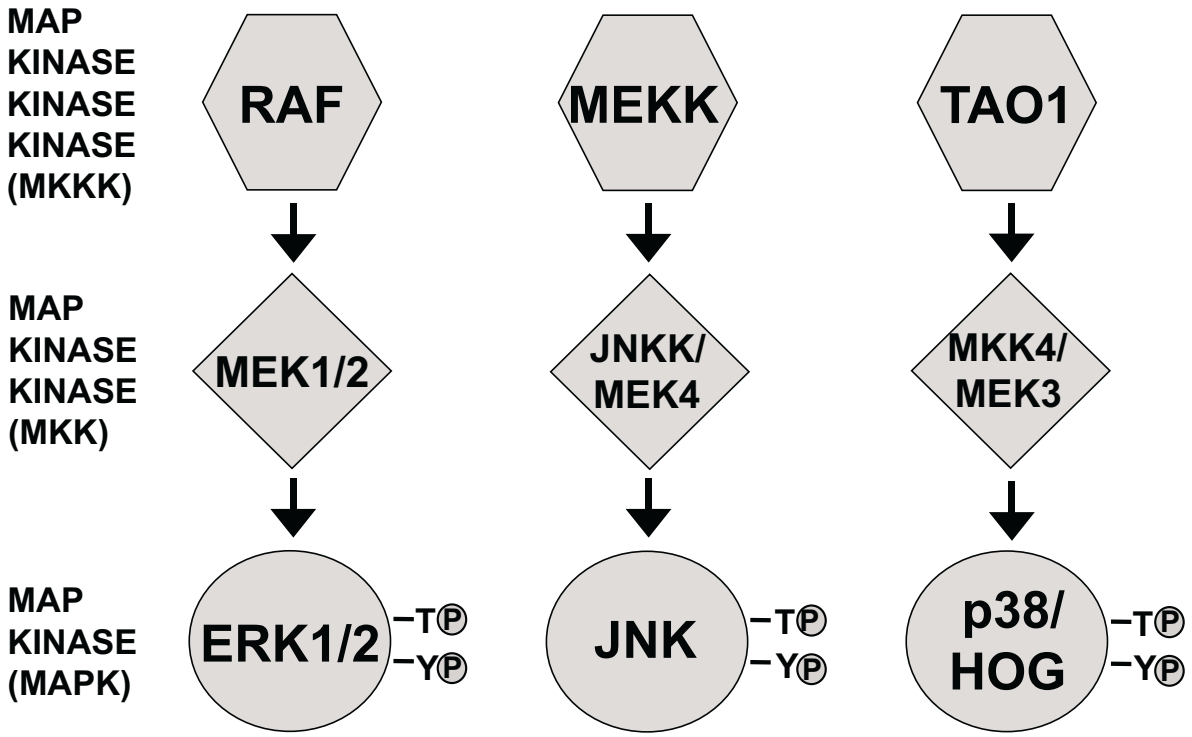

Fig. 1. A superfamily of MAPK signaling cascades. The MAPK cascade is characterized by a core of three kinases: a MAP kinase kinase kinase (MKKK), a MAP kinase kinase (MKK), and a MAP kinase (MAPK). The MKKK typically phosphorylates and activates the MKK on serine and/or threonine amino acids. The MKK is a dual-specific kinase that phosphorylates its MAPK on both a tyrosine and a threonine amino acid; this dual phosphorylation event is required for activation of the MAPK and is considered a signature of the MAPK cascade. In this paper, we have focused on the ERK cascade, which consists of Raf, MEK, and ERK. Two other distinct cascades have been established: the JNK MAPK cascade and the p38/HOG MAPK cascade.

MAPK cascade (Fig. 1). The prototypic member is, as just described, the ERK MAPK cascade, involving Raf, MEK1/2 and ERK. Another well described MAPK cascade in mammalian cells is the stress-activated protein kinase pathway, involving MEKK (a MAP kinase kinase kinase), MEK4/JNK kinase (JNKK, a dual-specific MAP kinase kinase) and *c-jun* N-terminal kinase (JNK, a MAP kinase). A third, more recently described MAPK pathway is that of p38 HOG (a MAP kinase), which is activated by the dual-specific kinase MEK3/MKK4 (a MAP kinase kinase). The activator of MEK3, a presumed MAP kinase kinase kinase, has been tentatively identified as TAO1 (Hutchison et al. 1998).

Importantly, distinct stimuli appear to selectively trigger the activation of a particular MAPK cascade. Furthermore, although the components of these pathways are highly homologous, there appears to be little if any crosstalk across individual MAPK pathways. Thus, MEK1/2, for example, is thought to be dedicated strictly to the ERK MAPKs, and does not regulate either JNK or p38 HOG (Lange-Carter et al. 1993, Minden et al. 1994). Conversely, the ERK MAPKs are activated only by MEK 1/2, and are thus not substrates for MEK4/JNKK or MEK3/MKK4.

INACTIVATION OF MAPKs

Interestingly, inactivation of these related cascades also appears to involve phophatases dedicated to a particular MAPK. It was initially demonstrated that the ERK MAPKs can be dephosphorylated and inactivated *in vitro* by phosphatase 2A, a general phosphoserine/phosphothreonine phosphatase, or CD45, a phosphotyrosine-specific phosphatase (Anderson et al. 1990). Whether either of these phosphatases is a physiological regulator of ERK MAPKs, however, is now debated. More recent evidence suggests that each MAPK cascade is specifically regulated by a dual-specific threonine/tyrosine phosphatase (Sun et al. 1993, Ward et al. 1994, Chu et al. 1996, Muda et al. 1996, Camps et al. 1998). Thus, at present, the leading model of MAPK cascade regulation is that each MAPK is regulated by a dual-specific MAPK kinase and a dual-specific MAPK phosphatase. Regulation of MAPK activity, then, would most likely reflect the balance of activity of these two enzymes. Finally, it is possible that persistent MAPK activation is prevented by an increase in the activity of these MAPK-specific phosphatases. In many cell types, activation of the MAPK cascade elicits the transcription and translation of these dual-specific MAPK phosphatases (Sun et al. 1993). Thus the MAPK cascade can initiate its own inactivation via this system of negative feedback.

SELECTIVE ACTIVATION OF ERK ISOFORMS

In most cell types, stimuli that trigger the MAPK cascade activate both ERK1 and ERK2 (reviewed in: Seger and Krebs 1995). In several studies, however, it has been observed that a selective activation of ERK2 can occur; for example studies of the MAPK cascade in neurons demonstrate that PKC and glutamate receptor activation lead to the activation of ERK2 but not ERK1 (Bading and Greenberg 1991, Fiore et al. 1993, English and Sweatt 1996, English and Sweatt 1997). Though this differential regulation is not understood, these findings suggest that neurons, and perhaps other cell types may utilize unique biochemical mechanisms for controlling ERK1 and ERK2 activation.

As previously described, the most likely regulator of ERK activation in neurons is MEK1. Interestingly, experiments using purified MEK1, ERK1 and ERK2 have demonstrated that MEK1 phosphorylates and activates both ERK1 and ERK2 with similar kinetics (Zheng and Guan 1993). This observation suggests that the selective activation of ERK2 in neurons is not due to an ERK2-specific MEK. One possibility is that ERK1 and ERK2 have distinct subcellular distributions in neurons and that the population of MEK1 activated is compartmentalized with ERK2. There is, however, at present little evidence to suggest differential localization in neurons.

The leading hypothesis for selective ERK2 activation is the presence of putative scaffolding proteins that organize the three-kinase module of the MAPK signaling cascade (Zanke et al. 1996). This model has been suggested as a possible mechanism by which the various members of the superfamily of MAPK signaling cascades remain independent (see Fig. 1). For example, the enzyme MEK kinase (MEKK) was so named because it phosphorylated MEK *in vitro* (Lange-Carter et al. 1993). Subsequent experiments, however, revealed that MEKK is not a physiological regulator of MEK and thus ERK MAPKs, but rather a MAP kinase kinase kinase devoted to different MAPK cascade, the JNK MAPK pathway (Minden et al. 1994). The scaffold model postulates that crosstalk among these MAPK cascades is prevented by regulatory scaffolding proteins that govern the interactions of particular MAPKs and their upstream activators.

Thus, a putative ERK MAPK pathway scaffolding protein might selectively bring together Raf, MEK and ERK, while a putative JNK MAPK pathway scaffolding protein might organize MEKK, JNKK and JNK. A simple extension of this model for explaining selective ERK2 activation would be the hypothesis of a specific ERK MAPK cascade scaffolding protein that selectively binds Raf, MEK and ERK2. Most of the evidence for the existence of such scaffolding proteins comes from studies of MAPK cascades in yeast (Yablonski et al. 1996), although recent reports have identified a potential ERK MAPK scaffolding protein in PC12 cells (see: Elion 1998).

LOCALIZATION OF MAPKs

As ERK MAPK was initially identified as a downstream effector of growth factor receptor signaling, the MAPK cascade has been most intensely studied in the context of mitotic cell regulation. In fact, of the more than 50 different oncogenes that have been identified, the majority encode proteins which participate in the growth factor receptor-MAPK pathway (Cantley et al. 1991, Seger and Krebs 1995). It is therefore interesting to note that MAPKs are actually most abundantly expressed in postmitotic neurons of the developed nervous system (Boulton et al. 1991).

ERK1 and ERK2 are highly expressed in the adult mammalian central nervous system; ERK3, however, appears to be expressed in the developing CNS, but is poorly expressed in mature hippocampal neurons (Boulton et al. 1991, Fiore et al. 1993). Immunohistochemistry studies have demonstrated that, in the rat CNS, ERK2 is expressed primarily in neurons, and is localized to the soma and dendritic tree (Fiore et al. 1993). Localization studies of ERK1 have not been reported.

Seven isoforms of MEK have been identified to date: MEK1 through MEK7. However, localization studies have only been performed with MEK1 and 2. MEK1 is mostly highly expressed in the adult mammalian CNS and appears to be the primary regulator of ERK1 and ERK2 in mature neurons (Crews et al. 1992). MEK2 is expressed in the developing CNS, but does not appear to be present in the mature CNS (Brott et al. 1993). The other MEKs are involved in the JNK and p38 MAPK pathways. For instance, MEK4 is the MKK for JNK, while MEK3 and MEK6 can both act as the MKK for p38 (Efimova et al. 1998, Hutchison et al. 1998).

Roles for MAPK in addition to Mitogenesis

Though little is known of either the regulation or physiologic roles of the Raf/MEK/ERK cascade in post-mitotic neurons, several lines of evidence suggest that the ERK MAPK pathway is involved in regulating neuron development, differentiation, and synaptic strength. Initial insights into the possible regulation of the MAPK cascade in neurons came from studies using cell cultures of embryonic neurons or pheochromocytoma cells (PC12 cells). For example, several studies have demonstrated that pharmacological stimulation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor leads to the activation of ERK2 in both cortical and hippocampal neurons in culture (Bading and Greenberg 1991, Kurino et al. 1995, Wang and Durkin 1995), or in hippocampal slices (English and Sweatt 1996, 1997). In addition, stimulation of metabotropic glutamate receptors elicits ERK2 activation in cortical neurons (Fiore et al. 1993). Finally, stimulation of protein kinase C produces a robust activation of ERK2 in both hippocampal and cortical neurons (Stratton et al. 1989, Fiore et al. 1993, English and Sweatt 1996, 1997).

THE ROLE OF THE MAPK CASCADE IN SYNAPTIC PLASTICITY

The first evidence that the MAPK cascade is involved in LTP came from English and Sweatt (English and Sweatt 1996). The authors first showed that ERK2 is activated in hippocampal area CA1 by NMDA receptor stimulation in the hippocampus. The authors then showed that LTP-inducing high frequency stimulation of the Schaffer-collateral inputs to area CA1 led to the activation of ERK2 in area CA1. This activation was NMDA-dependent and not observed with test stimulation. In a subsequent paper, the authors showed that ERK2 is required for the induction of stable long-term LTP (English and Sweatt 1997). Here the authors made use of a specific inhibitor of ERK activation, the compound PD098059. This chemical is a specific allosteric inhibitor of MEK, the kinase upstream of ERK2 (Alessi et al. 1995, Dudley et al. 1995). In 1999, O'Connor and colleagues found that ERK2 is involved in both NMDA-dependent (induced by HFS of the medial perforant path) and -independent (induced by application of the K^+ channel blocker tetraethylammonium chloride, TEA) LTP in the dentate gyrus of the rat (Coogan et al. 1999). These data have been recently con-

firmed using *in vivo* induction of LTP in the dentate gyrus (Rosenblum et al. 2000). Interestingly, ERK has also been shown to be involved in LTP at amygdala-insular cortex synapses (Jones et al. 1999); this pathway is thought to be involved in conditioned taste aversion (see below).

The role of ERK in synaptic plasticity seems to be a conserved function across species. Crow et al. (1998) found that ERK is involved in the long-term enhancement induced in sensory neurons of the invertebrate *Hermissenda* during classical conditioning. In these experiments, the conditioning consists of pairing light with high speed rotation. *Hermissenda* normally initiate locomotion in response to light; however, after pairing of the light with rotation, they exhibit suppression of locomotion. These experiments can also be performed *in vitro*, with presentation of light and serotonin to isolated eye preparations. The authors found that using *in vivo* or *in vitro* conditioning led to increased phosphorylation of ERK; using the *in vitro* conditioning, the ERK increase was blocked by pretreatment with the MEK inhibitor PD098059.

In another invertebrate species, *Aplysia californicus*, ERK has been shown to be required for long-term facilitation of sensory neurons during non-associative learning (Martin et al. 1997). The authors found that application of serotonin to the presynaptic sensory neuron, which leads to long-term facilitation of the connection between a sensory and motor neuron, leads to an increase in the nuclear localization of ERK. In addition, blocking ERK with injection of either anti-ERK antibodies or the MEK inhibitor PD098059 into the presynaptic cell led to a block of long-term facilitation of the EPSP recorded in the postsynaptic cell.

Thus, within four years of the first evidence of a role for MAPK in synaptic plasticity, abundant evidence exists that MAPK plays a role in LTP (both NMDA receptor dependent and independent) in area CA1 of the mammalian hippocampus, as well as in the mammalian insular cortex. Not only is MAPK is involved in mammalian synaptic plasticity, but evidence from two invertebrate species indicate that MAPK plays a role in synaptic plasticity across species.

THE ROLE OF THE MAPK CASCADE IN LTM

The first evidence that the MAPK cascade is involved in mammalian long-term memory was reported by Atkins et al. (1998), who showed that the MAPK cascade is required for fear conditioning in the rat. In these experiments, rats were taught to associate a foot shock with either a cue or a context. Later (up to 24 h), the extent of the animals' freezing in response to the cue or the context was quantified. Rats typically show robust freezing upon testing with either the cue or the context. Contextual fear conditioning is dependent on the hippocampus, while cued fear conditioning is dependent on both the amygdala and the hippocampus. The authors found that ERK2 phosphorylation was increased in rat hippocampus 1 h after training in both the cued and contextual paradigms. Importantly, the authors showed that this increase was not due to the handling of the rats or to the shock itself. The authors also showed that the MAPK cascade is required for fear conditioning. They found that injection of another MEK inhibitor, SL327, intraperitoneally led to a block of the freezing behavior of the rats upon testing in either the cued or the contextual conditioning paradigms. This MEK inhibitor is structurally and functionally similar to two other MEK inhibitors, PD098059 and U0126 (Favata et al. 1998). Interestingly, however, SL327 is able to cross the blood-brain barrier, and can thus be used as an inhibitor in the whole animal. Like PD098059 (English and Sweatt 1997) and U0126 (Winder et al. 1999, Selcher, Weeber and Sweatt, unpublished observations), SL327 blocks the induction of stable late-phase LTP in area CA1 of the hippocampus. These results showing the requirement of the MAPK cascade for long-term fear memory have been confirmed in the rat using intraventricular injection of the MEK inhibitor PD098059 (Schafe et al. 1999), and in the mouse using intraperitoneal injection of SL327 (Selcher et al. 1999). Subsequently, two laboratories have found that the MAPK cascade is involved in another form of hippocampal-dependent long-term memory, spatial memory (Blum et al. 1999, Selcher et al. 1999). To examine this involvement, both groups made use of the Morris water maze, an experimental paradigm that involves the use of a spatial search strategy to locate a platform hidden in a pool of opaque liquid. Selcher et al. (1999) found that intraperitoneal injection of the MEK inhibitor SL327 in mice led to impairments in two tests of the animals' spatial memory. First, the SL327-injected animals exhibited longer escape latencies; that is, their ability to find the hidden platform over time (six days) did not improve relative to vehicle-injected animals. Second, the SL327-injected animals were significantly worse than vehicle-treated controls in a probe trial on the last day of train-

ing; the probe trial is designed to test the animals' use of a spatial search strategy. Blum et al. (1999) found that intrahippocampal infusion of PD098059 also blocked acquisition of spatial memory in rats. Thus, compelling evidence now exists that the MAPK cascade is involved in two forms of hippocampal dependent long-term memory.

As mentioned above, it has been shown that the MAPK cascade is involved in long-term potentiation of the connections between the amygdala and the insular cortex. The insular cortex is thought to be the location of the consolidation of long-term taste memory; this memory is usually tested using the conditioned taste aversion paradigm (Hettinger and Frank 1992, Rosenblum et al. 1993). Berman et al. (1998) have shown that presentation of an unfamiliar taste leads to activation of ERK in the insular cortex within 30 min. Furthermore, they showed that microinjection of PD098059 into the insular cortex impairs conditioned taste aversion when tested up to 120 h after training. These data suggest that ERK is involved in insular cortex-dependent long-term memory.

Inhibitory avoidance training is a paradigm that involves learning an association between a footshock and an electric grid on the floor. After several trials in which the animal naturally steps down off a platform to explore the floor of the cage and is subsequently shocked, the animal learns to avoid stepping down off the platform. This type of learning has been shown to produce hippocampus-dependent long-term memory. In a series of papers by Walz et al. (1999, 2000), the MAPK cascade has been shown to be involved in this type of learning and memory. The authors found that injection of PD098059 into the entorhinal cortex or hippocampal area CA1 impaired retention of the avoidance when tested up to 6 h after training. Thus, not only does ERK appear to be involved in hippocampal-dependent long-term memory, evidence now also exists implicating ERK in insular and entorhinal cortex-dependent long-term memory.

CREB

The cAMP response element binding protein (CREB) is a transcription factor which binds to a specific DNA sequence, the cyclic AMP response element (CRE; consensus sequence 5'-TGACGTCA), found in the 5' flanking region of many genes (Montminy et al. 1986). It activates transcription of the downstream gene when phosphorylated at Ser133 (Chrivia et al. 1993). Ser133 is phosphorylated by PKA *in vitro* (Yamamoto et al. 1988), the pp90RSK family member RSK2 (Xing et al. 1996) and by calcium/calmodulin-dependent kinases including CaMKI (Sun et al. 1996), CaMKII (Dash et al. 1991, Sheng et al. 1991), and CaMKIV (Enslen et al. 1994, Matthews et al. 1994, Sun et al. 1994); CaMKII, though, is a poor activator of CREB as it also phosphorylates additional sites with negative effects (Enslen et al. 1994, Matthews et al. 1994, Sun et al. 1994, 1996). Phosphorylation of Ser133 recruits the CREB binding protein, CBP, to the complex and thereby promotes transcription (Chrivia et al. 1993). Many genes are activated by CREB, including other transcription factors such as *c-fos*, through which CREB signaling can indirectly activate an expanded range of genes (Goodman 1990, Herdegen and Leah 1998).

Evidence of a role for CREB in learning and memory

It has long been known that memory has distinct short-term and long-term components, with the latter being dependent on protein synthesis during learning (Agranoff et al. 1965, Barondes 1970, Davis and Squire 1984). In recent years evidence has accrued to indicate an important role for CREB in the long-term form of memory in several different systems (reviewed in: Yin and Tully 1996).

The first indication of CREB involvement in memory came from the observation that interruption of the interaction between CREB and the CRE blocks long-term facilitation in *Aplysia*, a mechanism underlying long-term sensitization of the gill- and siphon-withdrawal reflex (Dash et al. 1990). It was subsequently demonstrated that long-term facilitation-inducing stimuli produce an upregulation of CRE-driven genes that is dependent on phosphorylation of Ser133 in CREB (Kaang et al. 1993). CREB also plays an important role in *Drosophila* memory: expression of a dominant negative CREB blocks long-term memory while a CREB activator isoform facilitates the process such that long-term memory, which normally depends on multiple trial learning, can be induced by a single trial (Yin et al. 1994, 1995).

CREB is also implicated in mammalian memory. Mice with null mutation of αCREB and ΔCREB, the two major splice variants, have deficits in long-term but not short-term memory in several different tasks. These include the hippocampus-independent cued fear conditioning task and several hippocampus-dependent tasks, including context-dependent fear conditioning, the

Morris water maze test of spatial learning, and a more ethological task, social transmission of food preferences (Bourtchuladze et al. 1994, Kogan et al. 1997, see also: Gass et al. 1998).

Similarly, a selective effect on long-term memory in the water maze task was observed when antisense oligonucleotides to CREB were bilaterally injected into the hippocampi of adult rats (Guzowski and McGaugh 1997); this result is interesting in that the block of CREB function was spatially restricted to the hippocampus and free from any equivocality related to possible developmental effects of disrupting CREB function. Importantly, injection of the antisense DNA one day after training had no effect, indicating that CREB is selectively involved in the consolidation of learning, not in memory retention or retrieval.

Evidence of a role for CREB in LTP

The data indicating a role for CREB in learning and memory engender the question of its possible involvement in LTP. LTP-inducing stimuli do have effects on gene expression, altering the transcription of several different mRNAs in area CA1, including those for the transcription factor Zif/268, the subunit of CaMKII, and the growth factors BDNF and NT-3 (Mackler et al. 1992, Patterson et al. 1992, Roberts et al. 1996). The production of a transgenic mouse expressing a CRE-driven reporter construct has demonstrated that LTP is associated with an increase in CRE-mediated gene expression (Impey et al. 1996). Furthermore, CREB phosphorylation increases after the initiation of LTP both in area CA1 and in cultured hippocampal neurons (Deisseroth et al. 1996, Impey et al. 1996). Recently, immunocytochemical techniques have been used to show that CREB phosphorylation increases in the dentate gyrus of the rat 24 h after *in vivo* high frequency stimulation of the perforant path (Schulz et al. 1999). These lines of evidence all point to the involvement of CREB in hippocampal synaptic plasticity.

MAPK REGULATION OF CREB

It is well known that the regulation of cell division and proliferation in mitotic cells involves the regulation of gene expression by the MAPK cascade. However, the regulation of gene expression by the MAPK cascade in the context of new protein synthesis in post-mitotic neurons of the central nervous system is just beginning to be investigated. As mentioned above, we and Impey et al. (English and Sweatt 1996, 1997, Impey et al. 1998) have shown that in hippocampal area CA1, the MAPK ERK plays a role in late-phase LTP, which is known to be dependent on new protein synthesis. Additionally, it has been shown that the phosphorylation of the transcription factor CREB increases after stimulation procedures that generate long-lasting LTP (Impey et al., 1996). We therefore hypothesized that in hippocampal area CA1, ERK might regulate the phosphorylation of CREB.

To address this question, it was necessary that we first formulate a reliable procedure for the activation of ERK. In contrast to other kinases known to be involved in LTP, such as PKA and PKC, ERK cannot be activated by the application of specific exogenous pharmaceutical agents. For instance, the PKA cascade can be activated by the application of forskolin, which activates adenylyl cyclase, and PKC can be activated directly by the application of phorbol esters; no such homologue exists for ERK. However, as describe above, ERK is activated by a series of kinases. We therefore first ascertained which of the kinases upstream of ERK we could activate by application of exogenous pharmaceuticals. English et al. found that in the hippocampal slice, activation of PKC with phorbol diacetate (a phorbol ester) led to an increase in the phosphorylation of ERK2 in area CA1 as determined using western blotting protocols with an antibody that recognizes phospho-tyrosine residues. We confirmed that finding using a more specific antibody that recognizes dually-phosphorylated, activated ERK2 (Fig. 2). We found that application of phorbol diacetate to hippocampal slices led to an increase in the phosphorylation of ERK2 in hippocampal area CA1 as determined using western blotting protocols with the antibody that recognizes dually phosphorylated ERK2. In contrast, application of an inactive analog, 4- -phorbol, did not lead to any increase in phosphorylation relative to control tissue. The phorbol ester-induced increase was blocked completely by the conjunctive application of the MEK inhibitor U0126. It is interesting to note that both Ras and Raf are substrates *in vitro* for PKC, raising the possibility that PKC feeds into the MAPK cascade at these levels (Marquardt et al. 1994). At present it is unclear whether PKC activates Raf-1 directly (Howe et al. 1992, Kolch et al. 1993, Marquardt et al. 1994) or indirectly through Ras (Nori et al. 1992, Wood et al. 1992). In addition, a recently described, neuronal-specific cytosolic tyrosine kinase has been shown to be activated downstream of PKC; this kinase, PYK2, can also elicit

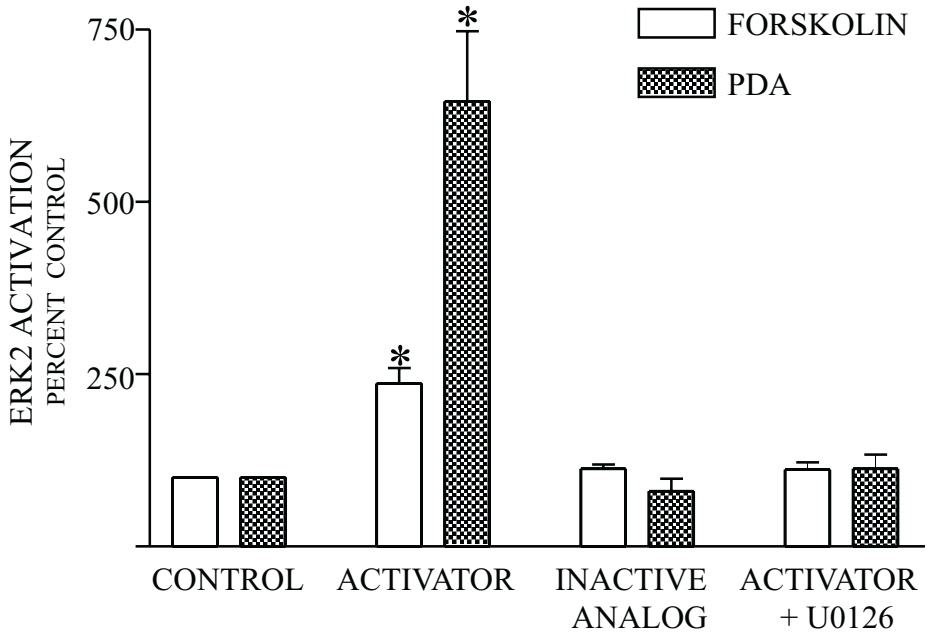

Fig. 2. Activation of ERK2 in hippocampal area CA1 *via* the PKA cascade and PKC. Summary data showing ERK2 activation in hippocampal area CA1 resulting from application of forskolin, an activator of the PKA cascade (left: 50 M with 100 M Ro20-172 for 10 min.) or phorbol diacetate, an activator of PKC (right: 10 M for 10 min.). Relative to vehicle-treated tissue (control), application of either forskolin or phorbol diacetate led to an increase in ERK2 activation (activator), as determined using western blotting techniques with a monoclonal antibody that recognizes phosphotyrosine residues (APT, clone 4G10, Upstate Biotechnology). In contrast, no increase was seen with application of the inactive analogs (inactive analog) dideoxyforskolin (left: 50 M for 10 min.) or 4- -phorbol (right: 10 M for 10 min.). The activator-induced increase was completely blocked by the MEK inhibitor U0126 (activator + U0126, 20 M for one h). These data were confirmed using two polyclonal antibodies, one that recognizes ERK phosphorylated at Tyr204, and one that recognizes ERK dually phosphorylated at Thr202 and Tyr204 (both, New England BioLabs).

MAPK activation, and thus potentially serve to link PKC to MAPK activation (Lev et al. 1995).

In the MAPK cascade the ubiquitously expressed Raf-1 is the main MAPKKK, although other Raf proteins including B-Raf can fill this role in the cells in which they are expressed. The respective interactions between the Raf proteins and MEK and between MEK and the MAPKs are very specific. Activation of the Raf-1-dependent pathway by growth factor receptors or other regulators is negatively regulated by PKA (Burgering et al. 1993, Graves et al. 1993, Sevetson et al. 1993, Chen and Iyengar 1994, Hordijk et al. 1994). One mechanism by which PKA exerts its inhibitory effect is through phosphorylation of Ser43 in Raf-1, which impairs its interaction with Ras, preventing its activation (Cook and McCormick 1993, Wu et al. 1993). In addition, PKA directly inhibits Raf-1 activity by phosphorylation of its catalytic domain (Hafner et al. 1994).

Given the known effects of PKA on Raf-1 signaling, the discovery that the cyclic AMP cascade can also activate ERK1 and ERK2 in PC12 cells was unexpected (Frodin et al. 1994, Okumura et al. 1994, Young et al. 1994). It has since been demonstrated that PKA is positively coupled to MAPK in some cell types, but that even in these cells, its effect on Raf-1 is inhibitory (Erhardt et al. 1995, Faure and Bourne 1995). The capacity for the cyclic AMP cascade to stimulate MAP kinase activity correlates with expression of a tissue-specific Raf isoform, B-Raf (Erhardt et al. 1995). B-Raf does not contain the Raf-1 Ser43 phosphorylation site (Sithanandam et al. 1990), suggesting one reason why it may be resistant to inhibition by PKA.

B-Raf expression, however, is not sufficient to confer the potential for PKA-stimulated ERK1 and ERK2 activation (Erhardt et al. 1995); the small GTP-binding protein, Rap1, is also required (Vossler et al. 1997). Rap1 is a Ras homologue that, like Ras, can activate B-Raf (Ohtsuka et al. 1996, York et al. 1998, Grewal et al. 2000). PKA phosphorylates Rap1 at Ser179 and leads to its activation (Kawata et al. 1989, Lapetina et al. 1989,

Hata et al. 1991, Altschuler et al. 1995). Thus, in cells expressing Rap1 and B-Raf, PKA leads to activation of MEK and its substrate MAP kinases *via* a pathway independent of Ras and Raf-1.

We examined the activation of ERK2 by PKA in rat hippocampal area CA1. We found that activation of adenylyl cyclase by application of forskolin to rat hippocampal slices resulted in secondary ERK2 activation in hippocampal area CA1. In contrast, no increase in ERK2 phosphorylation was seen with the inactive analog dideoxyforskolin. As was the case with PKC, the activator-induced increase was completely blocked by the conjunctive application of the MEK inhibitor U0126. We hypothesize that the foskolin-induced ERK2 phosphorylation occurs *via* Rap 1 and B-Raf. Indeed, we found in pilot experiments that both Rap 1 and B-Raf are expressed in the hippocampus.

Once we developed a robust and reliable method of activating ERK2 in hippocampal area CA1, we were able to investigate the regulation of CREB phosphorylation by ERK. We first performed *in vitro* experiments to determine if application of exogenous ERK could lead to increased CREB phosphorylation (Fig. 3). We incubated hippocampal homogenate with activated ERK in the presence of Mg^{2+} and ATP. In Western blot analyses, we found that immunoreactivity with a commercially available phospho-CREB antibody was increased relative to control homogenate not incubated with ERK. The increase was not blocked by conjunctive incubation of KN62, an inhibitor of CaMKII, or by IP_{20}, an inhibitor of PKA. Thus, *in vitro*, ERK leads to CREB phosphorylation, and this phosphorylation does not lie upstream of CaMKII or PKA. However, CREB lacks consensus sequences for ERK phosphorylation. It has

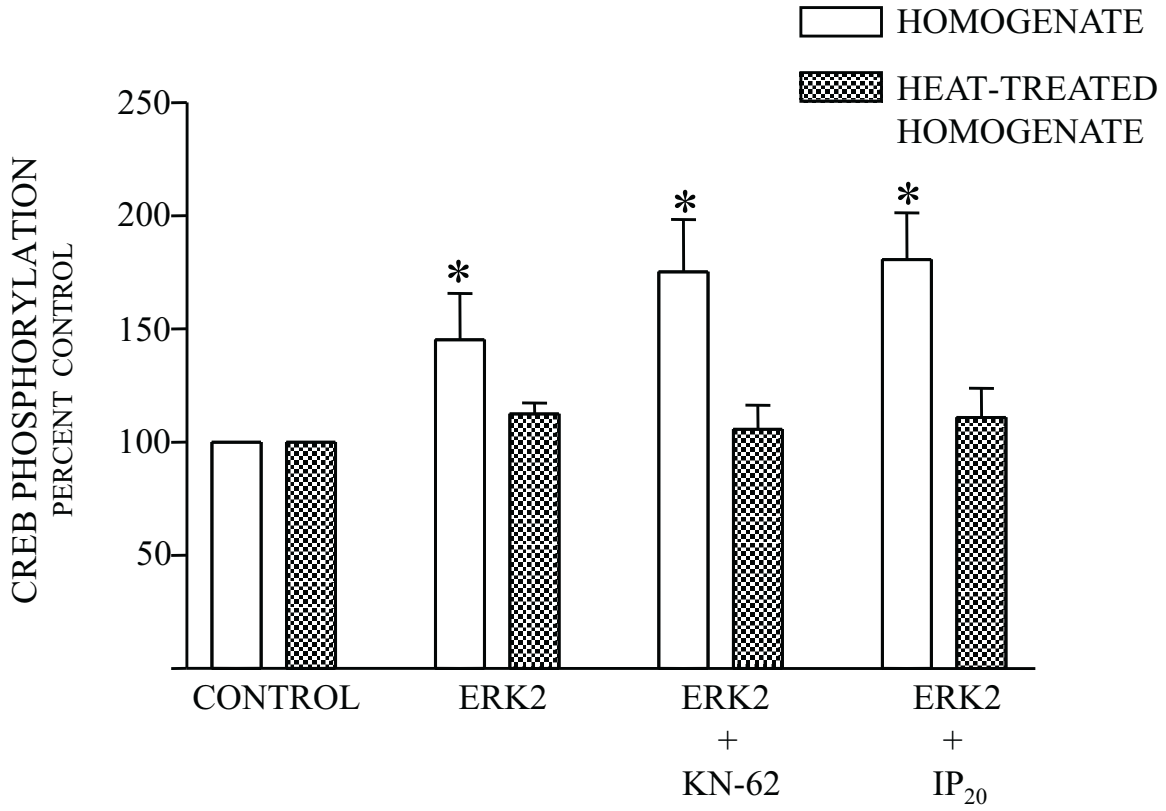

Fig. 3. *In vitro* phosphorylation of CREB by activated ERK2 does not involve PKA or CaMK. Summary data showing CREB phosphorylation in hippocampal homogenate after the addition of activated ERK2 (Stratagene) in the presence of ATP, Mg^{2+}, and phosphatase inhibitors. Left bars: incubation time for the reaction mixture was 30 min. Relative to control homogenate (control), the addition of activated ERK2 (ERK2) resulted in an increase in CREB phosphorylation as determined using western blotting techniques with a polyclonal antibody developed against rat CREB phosphorylated at Ser133 (Upstate Biotechnology). The ERK2-induced increase was not affected by addition of the CaMK inhibitor KN-62 or the PKA inhibitor IP_{20} (ERK2 + KN-62 and ERK2 + IP_{20}). Right bars: for the same conditions, hippocampal homogenate was boiled before the addition of activated ERK2. This heat-treatment eliminated the ERK2-induced phosphorylation of CREB in all conditions.

been shown that the ERK effector RSK2 is associated with increased phosphorylation of CREB in rat nuclear hippocampal extracts (Impey et al. 1998). In agreement with that data, we found in our experiments that boiling of hippocampal homogenate before the addition of activated ERK abolished the ERK-induced increase in CREB phosphorylation. Our data corroborate the necessity of a heat-sensitive intermediary such as RSK2 in the phosphorylation of CREB by ERK.

Once we determined that application of endogenous ERK led to increased CREB phosphorylation in hippocampal homogenate, we wanted to determine if activation of native ERK elicited increased CREB phosphorylation in rat hippocampal area CA1. We found that application of both forskolin, which activates the PKA cascade, and phorbol diacetate, which activates PKC, to hippocampal slices resulted in an increase in CREB phosporylation in area CA1 (Fig.4). However, application of the inactive analogs dideoxy forskolin or 4- -phorbol did not lead to any increase in CREB phosphorylation relative to control tissue. As mentioned above, PKA and PKC can directly phosphorylate CREB at Ser133. We therefore needed to determine if the PKA and PKC-induced increases in CREB phosphorylation were due to direct effects of these kinases or to the intermediate activation of ERK. We found that application of the MEK inhibitor U0126 in conjunction with forskolin or phorbol diacetate to rat hippocampal slices eliminated or attenuated, respectively, the activator-induced stimulation of CREB phosphorylation in area CA1. These data were surprising for two reasons. First, it has been shown that PKA can phosphorylate CREB *in vitro* (Montminy and Bilezikjian 1987); our data suggests strongly that in the hippocampus, PKA probably acts *via* ERK2 and RSK2 to phosphorylate CREB. This is in agreement with data from Impey et al. (1998) showing

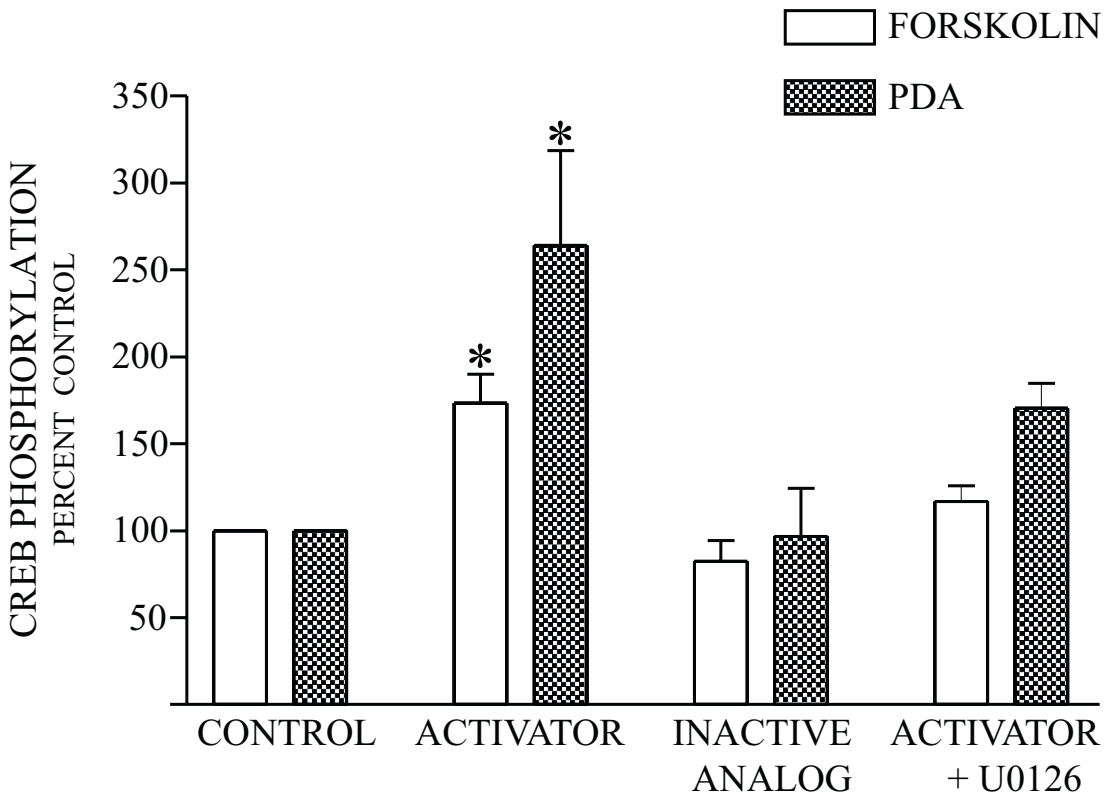

Fig. 4. Phosphorylation of CREB by ERK2 *via* PKA and PKC. Summary data showing CREB phosphorylation in hippocampal area CA1 following application of forskolin (left bar) or phorbol diacetate (right bar). Relative to vehicle-treated tissue (control), activation of the PKA cascade with forskolin or activation of PKC with phorbol diacetate resulted in increased CREB phosphorylation (activator), as determined using western blotting techniques with the phospho-CREB antibody described in Fig.3. In contrast, no increase was seen with application of the inactive analogs (inactive analog) dideoxyforskolin (left) or 4- -phorbol (right). The forskolin-induced increase was completely blocked by the MEK inhibitor U0126 (activator + U0126, left bar). The phorbol ester-induced increase was attenuated by U0126 (activator + U0126, right bar).

that Ca^{2+} stimulation of CRE-mediated transcription requires both PKA and ERK in both PC12 cells and cultured hippocampal neurons. Secondly, it had not been clear that PKC was a regulator of CREB; our data suggest that in the hippocampus, PKC may regulate CREB phosphorylation through the intervening kinases ERK and RSK2.

Overall, then these studies strongly support the idea that in LTP the MAPK cascade may contribute to CREB regulation of gene expression by both the PKA and PKC signal transduction systems.

DISCUSSION

Two recent reviews of the role of the MAPK cascade in synaptic plasticity (Impey et al. 1999, Orban et al. 1999) have ended by asking the question, is the regulation of CREB by MAPK important in neuronal synaptic plasticity? It is clear that both the MAPK cascade and the transcription factor CREB are important in both LTP and long-term memory. It has been shown that ERK is not only activated but required for LTP in area CA1 of the rat hippocampus and in the rat insular cortex, as well as for long-term synaptic plasticity in two invertebrate species. CREB phosphorylation has also been shown to increase after the induction of LTP in rat hippocampal area CA1. Similarly, ERK has been shown to be involved in several forms of hippocampus-dependent memory, as well as in taste memory mediated by the insular cortex. CREB has been implicated in memory in several species, including *Aplysia*, *Drosophila*, mouse and rat. Here, we have presented evidence that in rat hippocampal area CA1, activation of the MAPK cascade leads to CREB phosphorylation (Fig. 5).

We first showed that CREB is a downstream effector of ERK2 *in vitro*. We next examined the phosphorylation of CREB in response to ERK2 activation. We found that using activation of PKC or PKA in the hippocampus is a reliable method of activating ERK2 in hippocampal area CA1. Using this method, we demonstrate that activation of ERK2 in the hippocampus leads to increased CREB phosphorylation in hippocampal area CA1. Thus, it seems likely that, in answer to the question asked at the beginning of this discussion,

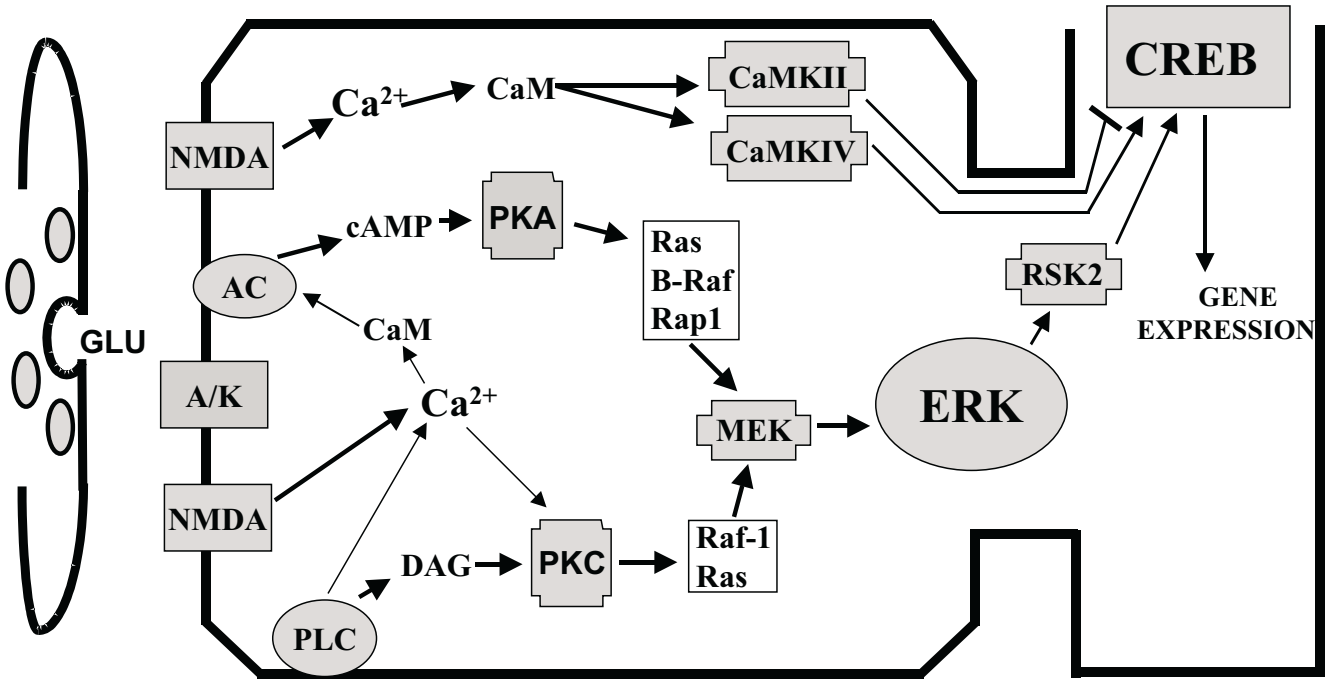

Fig. 5. Model for the regulation of CREB by ERK2. A presynaptic cell and its postsynaptic cell are depicted. Stimulation of NMDA receptors leads to an influx of Ca^{2+}. Ca^{2+} can then activate several second messenger systems, including PKC, PKA and CaMK. While CaMKs are thought to phosphorylate CREB directly, we hypothesize that PKA and PKC lead to CREB phosphorylation *via* the MAPK cascade and RSK2. Phosphorylation of CREB then leads to a change in gene expression; these changes in gene expression are postulated to underlie changes in synaptic plasticity at the cellular level.

CREB is a substrate for MAPK regulation during synaptic plasticity.

It is interesting to note that while we have found that PKA and PKC regulation of CREB occurs *via* ERK and RSK in the hippocampus, Ser133 is also a consensus sequence for other kinases including PKA and PKC themselves. This knowledge introduces the alternative hypothesis that PKA or PKC can phosphorylate CREB directly rather than through intervening kinases. No direct evidence to support this hypothesis is available; however, it is known that PKA and PKC can phosphorylate CREB *in vitro* (Yamamoto et al. 1988). In addition, CREB phosphorylation has been shown to increase after forskolin stimulation in PC12 cells (Montminy and Bilezikjian 1987). However, the pharma ceuticals have only recently become available to even allow the consideration that other kinases such as ERK may intervene between PKA or PKC and CREB. It is known that PKA, RSK and ERK can all translocate to the nucleus in PC12 cells (Hagiwara et al. 1993, Impey et al. 1998). The translocation of ERK has also been shown in other cells types such as COS-7 cells, HeLa cells, hamster fibroblasts, and *Aplysia* sensory neurons (Chen et al. 1992, Gonzalez et al. 1993, Lenormand et al. 1993, Impey et al. 1998). It has been suggested by Impey et al. (1998) that PKA may regulate the translocation of ERK to the nucleus. These authors found that inhibition of the PKA cascade led to a decrease in the nuclear localization of ERK in both PC12 cells and cultured hippocampal neurons. This inhibition also led to a decrease in the activation of nuclear RSK2, but not cytosolic RSK2. Thus, one model of ERK regulation of CREB might involve PKA activation with subsequent activation and trans location of ERK to the nucleus, where it can phosphorylate RSK2, which can then phosphorylate and activate CREB.

Innumerable studies have been conducted to examine the involvement of either PKC or PKA activity in cellular function, with areas of intense focus being regulation of gene expression, direct regulation of ion channels and hormone receptors, and control of metabolism. In many published experiments, activators of either PKC or PKA have been found to produce a physiologic effect upon the channel/receptor/enzyme studied. A conclusion has often been made that direct PKC/PKA phosphorylation mediates the observed effect, even in the absence of evidence demonstrating a direct phosphorylation. The demonstration that stimulation of either PKC or PKA can lead to activation of the MAPK cascade, coupled with the potentially novel targets of MAPK, suggests that many such conclusions should be reevaluated for the potential involvement of the MAPK cascade. Thus, while it is clear that both PKC and PKA can directly phosphorylate and regulate a variety of receptors, ion channels, etc., it remains a reasonable and attractive hypothesis that many of the physiologic effects of PKC and PKA activation in various cell types in part are secondary to PKC/PKA-mediated activation of MAPK.

It should be noted that although we have focused on the regulation of CREB by ERK, other nuclear targets have been shown to be effectors of the MAPK cascade. These include c-Fos, c-Jun, c-Myc, Elk-1, TAL-1, C/EBP , and ATF-2 (reviewed in: Davis 1993, 1995). Chief among these is Elk-1, which when phosphorylated at multiple sites by MAPK cooperates with serum response factor to drive transcription from serum response element (SRE)-controlled genes (Treisman 1996, Xia et al. 1996, Kaminska et al. 1999). Several of these substrates potentially may be regulated in synaptic plasticity (Kaczmarek 1992, 1993). For instance, recent findings suggest that neuronal excitation produced by kainate injection intraperitoneally may lead to an increase in the transcription of genes driven by AP-1, a transcription factor composed of Fos and Jun proteins (Jaworski et al. 1999). Here, the authors found that kainate injection resulted in tissue inhibitor of metalloproteinases (timp-1) mRNA accumulation in the hippocampus, as well as binding of AP-1 derived from the timp-1 promotor by AP-1 proteins, including c-Fos and c-Jun. Finally, the authors found that the timp-1 promoter is activated in dentate gyrus cells in culture after application of glutamate; this activation was shown to depend on the intact AP-1 site. From this and other data, it seems obvious that CREB is not the only transcription factor involved in LTP or LTM and probably not the only transcription factor regulated by ERK in synaptic plasticity.

A final issue to be examined is the role of CREB in synaptic plasticity. That is, what genes are being turned on by CREB phosphorylation (that occurring *via* ERK or other kinases)? This is an area of intense research, and the answers will hopefully be forthcoming with the advance of new genetic techniques.

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