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

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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.

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 (1,2). However, in many cases, the actions of these two second messengers involve slower, long-term changes in gene expression (3,4). 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) (5). Regulation of this protein may be important in mediating changes in synaptic plasticity and neuronal survival (6-8).

CREB binds as a dimer to a conserved cyclic-AMP response element (CRE) found in the promoters of numerous eukaryotic genes (4). Phosphorylation of serine-133 is a critical event in CREB activation (9), which induces an increase in CREB transactivation potential by allowing the recruitment and binding to co-activators such as CREB-binding protein (CBP) (10,11). Early studies identified PKA as a major physiological kinase responsible for Ser-133 phosphorylation (12). 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 (13). Subsequent studies have identified additional CREB kinases, including members of the calcium/calmodulin-dependent kinase (CaMK) family (14-18) and the extracellular signal-regulated kinase (ERK)-stimulated RSK and MSK kinases (19-21). 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 (5). 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 (11,22-28). 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 mechanism by which cAMP and calcium influx may stimulate ERKs in neuronal cells (29,30). 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 (29-31). 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 (30,32,33). 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.

EXPERIMENTAL PROCEDURES

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.

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% CO₂ prior to harvesting. NIH3T3 and Hek293 cells were maintained in DMEM plus 10% fetal calf serum. For luciferase assays and western blotting, cells were maintained in low serum (0.2% fetal calf serum) media for 16 hours at 37° C in 5% CO₂ prior to treatment with various reagents. All inhibitors were added 20 minutes before treatment.

Plasmids and Transfections - Fifty percent-confluent PC12 cells were co-transfected with the indicated cDNAs using a Superfect transfection kit (Qiagen) according to the manufacturer instructions. Hek293 and NIH3T3 cells were transfected using Lipofectamine (Gibco BRL) according to the manufacturer instructions. The vector pcDNA3 (Invitrogen Corp.) was added to each set of transfections as appropriate to ensure that each plate received the same amount of DNA. Following transfection, cells were allowed to recover for twenty-fours before being maintained in low serum media and
treated, as described. The following plasmids were used with the amounts used per 60mm dish indicated in brackets: PKI (1 or 3\(\mu\)g), GAL4-CREB (2\(\mu\)g), catalytic subunit of PKA (cPKA; 4\(\mu\)g), Rap1GAP1 (2 or 4\(\mu\)g), 5XGal-E1b-TATA-luciferase (gal-luciferase, 2\(\mu\)g), 5XCRE-luciferase (1\(\mu\)g), Fos-luciferase, (1\(\mu\)g), CREB-DIEDML (5 \(\mu\)g), GAL4-CREB-DIEDML (2 \(\mu\)g).

**Western blotting** - Cells were treated for appropriate times as indicated and then harvested in boiling SDS sample buffer. Cell lysates were boiled for additional five minutes and proteins resolved by SDS-PAGE. Western blotting with phospho-specific and phosphorylation state-independent antibodies was performed as per manufacturer instructions.

**Immunofluorescence assays** - PC12 cells were treated as described and after the indicated times fixed in paraformaldehyde. Following permeabilization in methanol and blocking in 5\% normal goat serum (NGS), cells were incubated in primary antibody (1:2500 in 5\%NGS) overnight at 4\(^\circ\)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 (34). 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 (Fig. 1). Similarly, elevation of intracellular cAMP levels by treatment with the adenylate cyclase activator, forskolin, also stimulated transcription (Fig. 1). In both cases, the increase in CRE-dependent transcription was reversed by either co-transfection of the selective PKA inhibitor, PKI (Fig. 1A), or pre-treatment with the selective MEK inhibitor, PD98059 (Fig. 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 (29,30). 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 (35). 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 (36). 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 3). In contrast, the MEK inhibitor PD98059 blocked KCl-, but not forskolin-induced phosphorylation (Fig. 3). We also examined CREB phosphorylation by immunofluorescence. Both KCl and forskolin induced an increase in phospho-CREB immunoreactivity (Fig. 4). These effects were reversed by transfection of the selective PKA inhibitor, PKI (Fig. 4B). Furthermore, as we observed with the western blots, PD98059 only blocked the increase CREB phosphorylation induced by KCl (Fig. 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 (14,37,38). 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 (Fig. 5). As observed with the CRE-luciferase reporter, these actions were reversed by the MEK inhibitor, PD98059 (Fig. 5A, B). Inhibition of Rap1 activity by co-transfection of Rap1GAP1 also prevented forskolin-mediated stimulation of GAL4-CREB activity (Fig. 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 (30). 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 (Fig. 6A). Moreover, these effects were reversed by PD98059 (Fig. 6A). In contrast, the phosphorylation of CREB Ser-133 induced by forskolin and isoproterenol was not blocked by PD98059 (Fig. 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 (30). Transfection of B-Raf into these cells allows PKA to stimulate ERKs (30). In the absence of B-Raf, both forskolin and cPKA were able to activate CRE-dependent
transcription (Fig. 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 (Fig. 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 (Fig. 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.

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(39). The resulting molecule allows constitutive, phosphorylation-independent binding to the co-activator CBP (39). Transfection of this CREB-DIEDML into F9 teratocarcinoma cells was previously shown to lead to constitutive stimulation of CRE-dependent transcription (39). We found that in PC12 cells, CREB-DIEDML also stimulated both CRE- and Fos-dependent transcription in an extracellular-signal-independent manner (Fig. 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 ((39) and Fig. 8B). Forskolin treatment potentiated GAL4-CREB-DIEDML activity further (Fig. 8B). Interestingly, as observed with the
GAL4-CREB protein, this stimulation by forskolin was reversed by PD98059 (Fig. 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 domain are unresponsive to calcium influx (14,38). 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 (Fig. 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 (5). 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 (17). 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 (21). PKA was also required for regulating the nuclear translocation of ERK/RSK2, a prerequisite for CREB phosphorylation (21). 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 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 (29). A similar pathway may also be used in hippocampal cells (29,40). 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 (4). 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 (41,42). 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 (11,22-28). 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 (39). Importantly, this mutant can bind CBP and stimulate CRE-dependent transcription in a phosphorylation-independent manner (39). 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 (27,28,43). Furthermore, PKA has been shown to augment GAL4-CBP function even when the sole consensus PKA phosphorylation site in CBP is mutated (11,44). 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 (45). In contrast, no effect of PKA was seen in COS-7 cells that express little or no B-Raf (45).

Some reports have suggested that stimulation of Ras-ERK signaling cannot increase CBP activity (22,24). In particular, Ras-dependent activation of RSK2 may actually antagonize CBP-dependent transcription (46). 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 (47,48), 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 (49,50), STATs (51), nuclear hormone receptors (52). 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 (53). 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 (54,55). 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 (56). 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 (5,57), we propose that activation of Rap1-ERK signaling may be important in mediating these neuronal events.
FIG 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.

FIG 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.
FIG 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).

FIG 4. **Depolarization- and forskolin–dependent stimulation of phospho-CREB immunofluorescence.** (A) PC12 cells were transfected with either empty vector or PKI, and GFP as a marker, as indicated. Following stimulation with either KCl (60 mM) 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).

FIG 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 (50µM) as indicated and then stimulated with either the cAMP analog, 8-CPT-cAMP (175µM) or forskolin (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) 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 (50µM) 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 (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.

FIG 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.

FIG 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 (10µM) 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.

**FIG 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 (58) (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.

**FIG 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. A similar step may also be utilized by calcium signaling.
REFERENCES


(A) fold increase CRE-luciferase

- **KCl**
  - 0: 0.1
  - 3: 6.5

- **Forskolin**
  - 1: 1.0
  - 3: 2.0

(B) fold increase CRE-luciferase

- **KCl**
  - 0: 0.5
  - 20: 4.0
  - 50: 8.0
  - 100: 1.0

- **Forskolin**
  - 0: 0.1
  - 50: 5.0
  - 100: 2.0

**µg PKI**
- 0: 0
- 1: 0
- 3: 0

**µM PD98059**
- 0: 0
- 50: 1.0
- 100: 0.5
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Calcium and Cyclic AMP signals differentially regulate CREB function via a Rap1-ERK pathway
Savraj S. Grewal, Daniel M. Fass, Hong Yao, Cindy L. Ellig, Richard H. Goodman and Philip J. S. Stork

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