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Neurotransmitter- and Growth Factor-Induced cAMP Response Element Binding Protein Phosphorylation in Glial Cell Progenitors: Role of Calcium Ions, Protein Kinase C, and Mitogen-Activated Protein Kinase/Ribosomal S6 Kinase Pathway

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To understand how extracellular signals may produce long-term effects in neural cells, we have analyzed the mechanism by which neurotransmitters and growth factors induce phosphorylation of the transcription factor cAMP response element binding protein (CREB) in cortical oligodendrocyte progenitor (OP) cells. Activation of glutamate receptor channels by kainate, as well as stimulation of G-protein-coupled cholinergic receptors by carbachol and tyrosine kinase receptors by basic fibroblast growth factor (bFGF), rapidly leads to mitogen-activated protein kinase (MAPK) phosphorylation and ribosomal S6 kinase (RSK) activation. Kainate and carbachol activation of the MAPK pathway requires extracellular calcium influx and is accompanied by protein kinase C (PKC) induction, with no significant increase in GTP binding to Ras. Conversely, growth factor-stimulated MAPK phosphorylation is independent of extracellular calcium and is accompanied by Ras activation. Both basal and stimulated MAPK activity in OP cells are influenced by cytoplasmic calcium levels, as shown by their sensitivity to the calcium chelator bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid. The kinetics of CREB phosphorylation in response to the various agonists corresponds to that of MAPK activation. Moreover, CREB phosphorylation and MAPK activation are similarly affected by calcium ions. The MEK inhibitor PD 098059, which selectively prevents activation of the MAPK pathway, strongly reduces induction of CREB phosphorylation by kainate, carbachol, bFGF, and the phorbol ester TPA. We propose that in OPs the MAPK/RSK pathway mediates CREB phosphorylation in response to calcium influx, PKC activation, and growth factor stimulation.

Key words: non-NMDA receptors; muscarinic receptors; basic fibroblast growth factor; ribosomal S6 kinase; transcription factor; oligodendrocytes

Calcium ions act as second messengers in the CNS (Clapham, 1995). Extracellular signals can increase intracellular calcium concentration ($[Ca^{2+}]_i$) and initiate signal transduction through different mechanisms. The activation of G-protein-coupled receptors or growth factor receptors can stimulate phospholipase C to produce inositol (1,4,5)-trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ triggers Ca^{2+} release from the endoplasmic reticulum (Berridge and Irvine, 1989), which is typically followed by capacitative entry of Ca^{2+} across the plasma membrane through store-operated channels (Clapham, 1996). Excitatory neurotransmitters can lead directly to Ca^{2+} influx through the opening of receptor channels permeable to this cation (Mayer and Miller, 1990). Finally, activation of receptor channels that depolarize the cell membrane can indirectly increase $[Ca^{2+}]_i$ through the gating of voltage-sensitive Ca^{2+} channels.

$[Ca^{2+}]_i$ increase can trigger various short- and long-term events, such as neurotransmitter release, synaptic plasticity, cell growth, survival, and death (Ghosh and Greenberg, 1995). It has been proposed that Ca^{2+} signals induce long-term cellular responses by regulating the function of several transcription factors, thus leading to new gene expression. In particular, analysis of heterologous gene promoters has indicated that cAMP response element binding protein (CREB) is a critical mediator of Ca^{2+}-dependent gene expression (Sheng et al., 1990). CREB constitutively binds to a short sequence in the promoter of several genes, the Ca^{2+}/cAMP response element (CaRE/CRE). Ca^{2+}, as well as cAMP and growth factor signals, activates CREB and promotes CRE-dependent transcription by inducing CREB phosphorylation at a specific amino acid residue, Serine-133 (Ser-133) (Sheng et al., 1991). CREB becomes phosphorylated during some forms of synaptic activity (Deisseroth et al., 1996) and is required for several learning processes and adaptive responses in the brain (Bourtchuladze et al., 1994; Maldonado et al., 1996).

Because of the complexity of Ca^{2+} signal transduction, it is still unclear how Ca^{2+} signals are propagated to the nucleus to regulate CREB Ser-133 phosphorylation. Ca^{2+} directly influences the activity of many key regulatory enzymes, such as Ca^{2+}-calmodulin-dependent kinases (CaMKs), protein kinase C (PKC), and Ca^{2+}-calmodulin-dependent adenylate cyclase, which in turn may activate cAMP-dependent protein kinase (PKA). All of these kinases phosphorylate CREB Serine-133 (Ser-133) *in vitro* (Yamamoto et al., 1988; Sheng et al., 1991). Similar to growth factor signals, Ca^{2+} can also activate the mitogen-activated protein kinase (MAPK) pathway (Finkbeiner and Greenberg, 1996),

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which involves Ras, raf kinases, MAP kinase kinase (MEK), MAPK, and p90 ribosomal S6 kinase (RSK). The physiological targets of the Ca^{2+}-activated MAPK pathway are still to be identified.

We have analyzed the Ca^{2+}-dependent signal transduction pathways leading to CREB phosphorylation in oligodendrocyte progenitor (OP) cells. OPs can be cultured as a pure and undifferentiated population of cells that maintain the developmental properties displayed *in vivo* (Dubois-Dalcq and Armstrong, 1992). OP cells co-express membrane receptors for various neurotransmitters and growth factors (Finkbeiner, 1993; Barres and Raff, 1994; Steinhauser and Gallo, 1996), and the role of these extracellular signals in oligodendrocyte development has been studied intensely (Barres and Raff, 1994; Gallo et al., 1996); however, the mechanism by which neurotransmitter and growth factor signals are integrated in these cells is still unclear. In the present study, we show that stimulation of ion channels, G-protein-coupled receptors, and tyrosine kinase receptors in OP cells leads to Ca^{2+}-dependent activation of the MAPK pathway, which can propagate membrane receptor signals to the nucleus by inducing CREB phosphorylation at Ser-133.

MATERIALS AND METHODS

Materials. Platelet-derived growth factor-AA (PDGF) and basic fibroblast growth factor (bFGF) were purchased from Upstate Biotechnology (Lake Placid, NY). Kainate, carbachol, 12-O-tetradecanoylphorbol-13-acetate (TPA), and forskolin were from Sigma (St. Louis, MO). PD 098059 was from New England Biolabs (Beverly, MA). KN-93 was from Seikagaku America (Ijamsville, MD). The acetoxymethylester of 1,2-bis-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA-AM) and fura-2 were from Molecular Probes (Eugene, OR). Anti-MARCKS and anti-GAP-43 antibodies were obtained from Alan Aderem (The Rockefeller University, New York, NY) and Rory Curtis (Regeneron Pharmaceuticals, Tarrytown, NY), respectively. Anti-CREB antiserum was purchased from Upstate Biotechnology. Phospho-specific CREB (Ser-133) antiserum (New England Biolabs) is raised against a synthetic phospho-Ser-133 peptide corresponding to residues 129 to 137 of human CREB. Phospho-specific MAPK (Tyr204) antiserum (New England Biolabs) is raised against a synthetic phospho-Tyr204 peptide corresponding to residues 196 to 209 of human ERK-1. Anti-calmodulin-dependent kinase (CaMK) II antibodies CBα-2 and CBβ-1 were from Life Technologies (Gaithersburg, MD). Anti-RSK antiserum was either purchased from Upstate Biotechnology or obtained as described previously (Chen and Blenis, 1990). Anti-Pan PKC antiserum (Upstate Biotechnology) is raised against a C-terminal peptide of PKCβII and cross-reacts with PKC α, βI, γ, and δ isozymes; its cross-reactivity with the atypical isoforms of PKC was not tested. Anti-v-H-Ras antibody Ab-1 (Oncogene Science, Cambridge, MA) reacts with H-, K- and N-Ras proteins. Anti-p70 S6 ($p70^{S6K}$) kinase antiserum is raised against the C-terminal region of the protein (Chung et al., 1992).

Cell culture and stimulation. Cortical OP cells were prepared from embryonic day 20 Sprague Dawley rats as described previously (Patneau et al., 1994; Gallo and Armstrong, 1995). Cells were grown for 2–4 d on polyornithine-coated plastic dishes (for biochemical experiments) or glass coverslips (for calcium-imaging experiments) in DMEM (Life Technologies)-N1 supplemented with 30% B-104 neuroblastoma cell-conditioned medium (Louis et al., 1992). The OP cultures contained >95% of LB1(anti-GD3)-positive cells, and ~3% of O4-positive pro-oligodendroblast (Gallo and Armstrong, 1995; Gallo et al., 1996). The culture medium was removed from OP cell cultures and replaced with DMEM 4–5 hr before stimulation. Stimulating agents and kinase inhibitors were added directly to the cell culture medium.

Calcium measurements. OP cells were incubated with 5 μM fura-2 AM for 20 min at room temperature, as described previously (Fatatis and Russell, 1992). Ca^{2+}-imaging experiments were performed as described previously (Yagodin et al., 1994).

Immunoblot analysis. After incubation with stimulating agents for the indicated periods of time, cells were washed twice with PBS, and total cell extracts were prepared as described by Ginty et al. (1993). OP cells ($5–7 \times 10^5$ cells in 35 mm plates) were lysed in 0.1 ml of boiling sample buffer (62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol), and boiled for 5 min. Protein extracts were electrophoresed on 10% polyacrylamide gels and transferred to Immobilion membranes (Millipore, Marlborough, MA). Blots were blocked with 4% BSA (Miles, Kankakee, IL) in a buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 (TBST) for 1 hr at room temperature, and then incubated overnight at 4°C with either anti-P-CREB (1:2000), anti-P-MAPK (1:2000), anti-RSK (Upstate Biotechnology; 2 μg/ml), or anti-Pan-PKC (1 μg/ml) antisera in TBST with 4% BSA. Immunoreactivity was visualized by chemiluminescence detection systems (ECL, Amersham, Arlington Heights, IL, or Phototope-Star, New England Biolabs). Films were scanned, and immunoreactivity was determined by densitometry (Microtek ScanWizard Plug-In, Redondo Beach, CA).

Cell labeling and immunoprecipitation. After a 2 hr starvation in DMEM, OP cells ($2–3 \times 10^6$ cells in 60 mm tissue culture plates) were incubated for 1 hr in phosphate-free DMEM and then metabolically labeled with [^{32}P]-orthophosphate (DuPont NEN, Boston, MA) (200 μCi in 1.5 ml of phosphate-free medium) for 2 hr before stimulation. After treatment, cells were collected in 0.5 ml of cold RIPA buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM EGTA, 50 mM NaF, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM [4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride], 10 μg/ml leupeptin, 10 μg/ml aprotinin) and drawn 10 times through a 0.22 gauge needle. Lysates were centrifuged at $40,000 \times g$ for 15 min at 4°C, and supernatants were incubated for 1 hr at 4°C with either anti-MARCKS (2 μl/tube), anti-GAP-43 antiserum (5 μl/tube), or the combination of CB-β-1 (1 μl/tube) and CB-α-2 (2 μl/tube) subunit-specific anti-CaMK II antibodies. Immune complexes were isolated using protein A Sepharose beads (50 μl; Zymed, San Francisco, CA). Immunoprecipitates were washed twice with buffer A (10 mM Tris, pH 8, 500 mM NaCl, 0.5% NP-40, 0.05% SDS), once with buffer B (10 mM Tris, pH 8, 150 mM NaCl, 0.5% NP-40, 0.05% SDS, 0.5% sodium deoxycholate), once with buffer C (10 mM Tris, pH 8, 0.05% SDS), solubilized in boiling SDS sample buffer for 5 min, and resolved on SDS polyacrylamide gels. Phosphoprotein levels were detected by autoradiography and quantified by using PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Determination of Ras GTP/GDP ratio. OP cells (4×10^6 cells in 100 mm tissue-culture plates) were starved in N1 medium for 1 d and then labeled metabolically with [^{32}P]-orthophosphate (DuPont NEN; 500 μCi in 3 ml of phosphate-free DMEM) for 4 hr. After treatment with stimulating agents, cells were lysed in 0.5 ml of a buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, and 2 μg/ml of anti-Ras antibody. Ras immunoprecipitation and GTP loading assays were performed essentially as described in Rosen et al. (1994).

Radiolabeled phorbol ester binding assay. OP cells (5×10^5 cells in 35 mm tissue-culture plates) were washed once with a balanced salt solution (BSS) (160 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 10 mM glucose, 0.1% fatty acid-free BSA), and incubated at 20°C for 10 min in 1 ml of BSS containing the designated treatment, and 1 nM phorbol-12,13-dibutyrate ([^{3}H]-PDBu, 20 Ci/mmol, DuPont NEN) (Vaccarino et al., 1991). Nonspecific binding was determined by adding 1 μM TPA to the incubation medium. In the Ca^{2+}-free medium, $CaCl_2$ was omitted from the BSS. Cells were then washed rapidly with ice-cold BSS and lysed with 0.1 M NaOH. Aliquots of the extracts were used for protein determination and liquid scintillation counting.

cAMP and kinase assays. Kinase assays for RSK and $p70^{S6K}$ kinase were performed as described previously (Chen and Blenis, 1990), using GST-S6 as a substrate (Fisher and Blenis, 1996). cAMP levels were assayed as directed by kit manufacturers (Amersham).

RESULTS

CREB Ser-133 phosphorylation by extracellular signals

CREB nuclear factor is constitutively expressed in cells of the oligodendrocyte lineage (Sato-Bigbee and Yu, 1993) (M. Pende and V. Gallo, unpublished data). We examined whether extracellular signals trigger CREB Ser-133 phosphorylation in OP cells, an event necessary for the transcriptional activating function of this protein (Gonzalez and Montminy, 1989). OP cells were treated with various stimulating agents, and cell extracts were immunoblotted with a phospho-specific antiserum (anti-P-CREB), which recognizes the 43 kDa CREB protein only when the Ser-133 amino acid residue is phosphorylated (Ginty et al.,

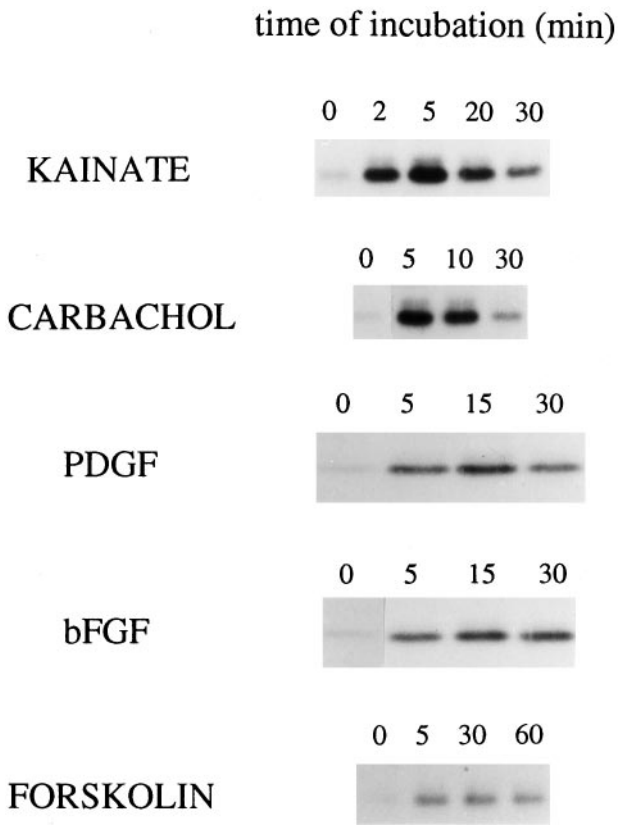

Figure 1. Activation of ligand-gated channels, G-protein-coupled and growth factor receptors in OP cells causes CREB phosphorylation with a different temporal pattern. Immunoblot analysis with an anti-P-CREB antiserum of extracts from OP cells incubated for the indicated periods of time with 300 μM kainate, 300 μM carbachol, 10 ng/ml PDGF, 10 ng/ml bFGF, and 50 μM forskolin.

1993). When OP cells were incubated for various periods of time with the non-NMDA glutamate receptor agonist kainate, the cholinergic agonist carbachol, or the growth factors bFGF and PDGF, a significant stimulation of CREB Ser-133 phosphorylation was observed (Fig. 1). Direct activation of PKC by the phorbol ester TPA also triggered a sustained CREB phosphorylation (data not shown), whereas increase of cAMP levels by forskolin led to only a moderate induction (Fig. 1). The effect of carbachol on CREB phosphorylation was found to be mediated by G-protein-coupled muscarinic receptors, because it was mimicked by subtype-selective agonist methacholine and was antagonized by atropine (data not shown). Immunoblot analysis with an anti-CREB antiserum that recognizes both the phosphorylated and unphosphorylated forms of CREB showed no change in total CREB protein levels after incubation with stimulating agents (data not shown).

Analysis of the kinetics of CREB phosphorylation in response to different stimuli indicated that kainate and carbachol elicited a rapid and transient phosphorylation of the nuclear factor, which peaked 5 min after receptor activation and then declined toward basal levels. In contrast, responses to the growth factors PDGF and bFGF displayed a slower onset, remaining constant for at least 30 min in the case of bFGF, and decreasing after 15 min in the case of PDGF. Moreover, the effects of PDGF and bFGF were not additive (data not shown), suggesting that the two growth factors lead to CREB phosphorylation through a common intracellular pathway. Taken together, these results indicate that in OP cells, as observed in other systems, the transcription factor CREB is a nuclear target for multiple signaling pathways that are initiated by activation of ion channels, as well as G-protein-coupled and tyrosine kinase receptors. The distinct kinetics of CREB phosphorylation in response to neurotransmitters and growth factors may account for differential regulation of gene expression by these two classes of extracellular signals.

Effect of kainate, carbachol, and bFGF on $[Ca^{2+}]_i$

We next asked at what level kainate, carbachol, and growth factor signaling pathways converge in OP cells to produce identical nuclear responses, i.e., CREB Ser-133 phosphorylation. Because all of these stimuli are known to alter Ca^{2+} homeostasis in OP cells (Hart et al., 1989; Cohen and Almazan, 1994; Holtzclaw et al., 1995; Meucci et al., 1996), we reasoned that Ca^{2+} might be a common second messenger necessary for signal transduction to the nucleus. Fura-2-based Ca^{2+} imaging experiments showed that stimulation of OP cells with kainate, carbachol, or bFGF produced intracellular Ca^{2+} responses that differed in amplitude and time course. Incubation of OP cells for 5 min with kainate elicited a large and persisting rise in $[Ca^{2+}]_i$ (Fig. 2*A*). This was caused by transmembrane Ca^{2+} influx, because it was prevented by removal of Ca^{2+} from the extracellular solution (Fig. 2*A*) and is mainly attributable to Ca^{2+} flowing through the kainate-gated ion channel itself (Fulton et al., 1992; Pende et al., 1994; Puchalski et al., 1994; Meucci et al., 1996). Cells treated with carbachol showed a transient $[Ca^{2+}]_i$ peak elevation followed by a sustained plateau that lasted during the entire period of agonist application (Fig. 2*B*). The peak phase was evoked either in normal external $[Ca^{2+}]$ (1.5 mM) or in nominally Ca^{2+}-free medium and therefore was attributable to Ca^{2+} release from intracellular stores (Simpson et al., 1995). Conversely, the plateau component was absent when carbachol-stimulated cells were perfused in a Ca^{2+}-free solution (Fig. 2*B*), indicative of a capacitative Ca^{2+} entry across the plasma membrane (Clapham, 1996) (P. Simpson and J. Russell, unpublished data). Finally, $[Ca^{2+}]_i$ increases in response to bFGF, alone or in combination with PDGF, were characterized by slow kinetics and extremely low amplitude (*filled circles* in Fig. 2*C* represent one of the largest Ca^{2+} responses to the growth factor). Although the majority of cells responded to kainate and carbachol (>95%; $n = 53$ for kainate, $n = 33$ for carbachol), a rise in $[Ca^{2+}]_i$ during bFGF exposure was detectable in only 22% of the cells analyzed ($n = 119$). Treatment of OP cells with bFGF in the absence of extracellular Ca^{2+} also evoked a response in only a small percentage of cells (16%, $n = 100$; Fig. 2*C* shows a recording from a cell that did not respond to bFGF in the absence of external Ca^{2+}).

To abolish agonist-evoked increases in intracellular Ca^{2+} levels (because of either Ca^{2+} release from intracellular stores or influx of the cation across the plasma membrane), we exposed OP cells to BAPTA, a very effective Ca^{2+} chelator (Tsien, 1980). Figure 2 shows that preincubation of OP cells for 45 min with the membrane-permeant BAPTA-AM strongly attenuated Ca^{2+} transients in response to kainate, carbachol, and bFGF, thus providing an effective tool for understanding the role of intracellular Ca^{2+} in signal transduction in OP cells (see below).

Ca^{2+}-dependence of CREB phosphorylation

We next studied whether interfering with intracellular Ca^{2+} transients affected the induction of CREB phosphorylation by neurotransmitters and growth factors. The omission of Ca^{2+} ions from

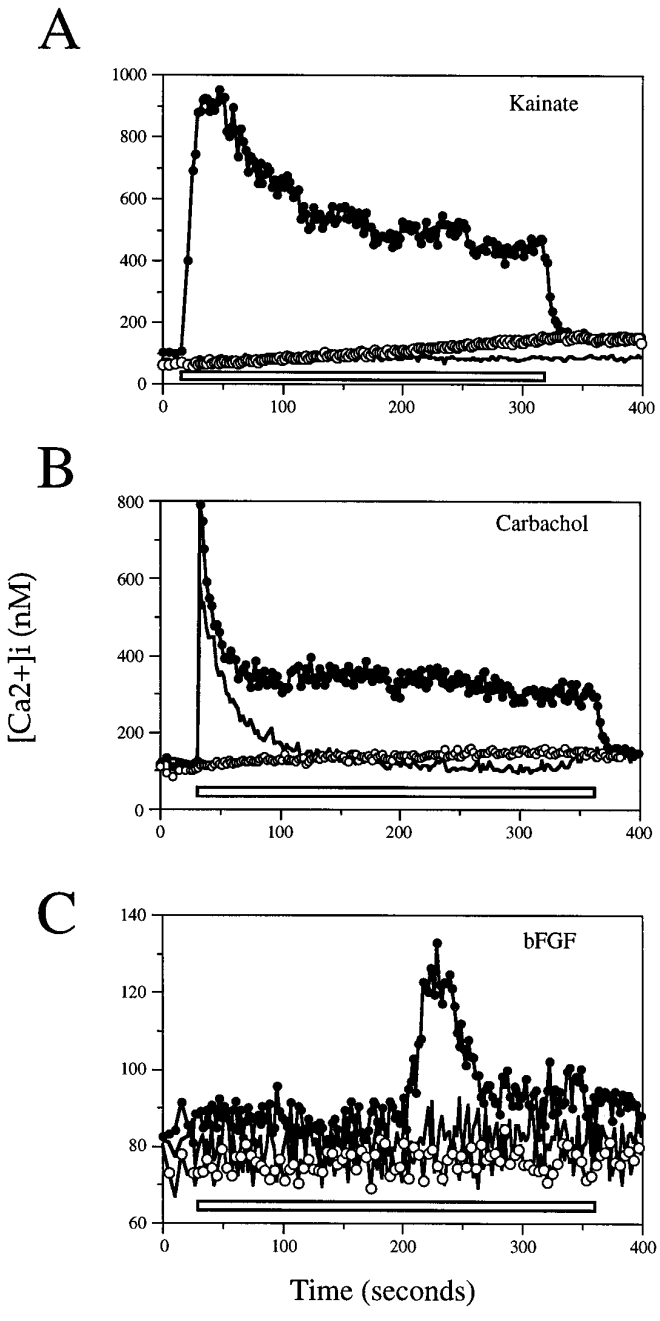

Figure 2. Fura-2 measurements of intracellular Ca^{2+} levels in response to kainate, carbachol, and bFGF. Representative traces recorded from individual OP cells during perfusion with 300 μM kainate (*A*), 300 μM carbachol (*B*), and 10 ng/ml bFGF (*C*). The period of agonist application (5 min) is indicated by the *bar* at the *bottom* of the traces. Incubation of OP cells with bFGF for a longer period of time (9 min) did not result in an increase in the percentage of responding cells (14%; $n = 63$). OP cells were incubated with the stimulating agents in the presence of 1.5 mM extracellular Ca^{2+} (*filled circles*), in nominally Ca^{2+}-free buffer (*no symbols*), or in a Ca^{2+}-containing buffer in the presence of 45 μM BAPTA-AM (*open circles*). Cells were treated with BAPTA-AM for 45 min before stimulation.

the extracellular medium completely abolished kainate-induced CREB phosphorylation (Fig. 3). The effect of carbachol was also strongly attenuated in the absence of extracellular Ca^{2+}, indicating that capacitative Ca^{2+} entry across the membrane is the major trigger of the signaling pathway leading to CREB phosphorylation

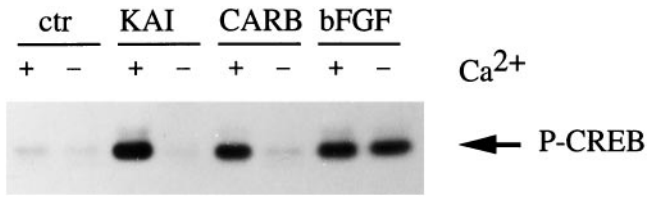

Figure 3. The effect of kainate and carbachol on CREB phosphorylation requires transmembrane influx of extracellular Ca^{2+}. Immunoblot analysis with an anti-P-CREB antiserum of extracts from OP cells treated as indicated (*KAI*: 300 μM kainate for 5 min; *CARB*: 300 μM carbachol for 5 min; *bFGF*: 10 ng/ml bFGF for 15 min). Cells were incubated with the stimulating agents in a balanced salt solution containing 160 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, in the presence (+ *lanes*) or in the absence (− *lanes*) of 2 mM $CaCl_2$. The 43 kDa CREB phosphoprotein is indicated by the *arrow*.

on muscarinic receptor activation (Fig. 3). The effect of bFGF was not influenced by the absence of extracellular Ca^{2+} (Fig. 3); however, chelation of intracellular Ca^{2+} by BAPTA not only reduced kainate- and carbachol-evoked CREB phosphorylation, it also inhibited growth factor signaling to CREB (Fig. 4*A*). These results suggest that the kainate-, carbachol-, and growth factor-activated pathways leading to CREB phosphorylation are all regulated, to some extent, by intracellular Ca^{2+}.

Activation of putative CREB kinases in OP cells

To investigate the Ca^{2+}-dependent pathways linking receptor activation with phosphorylation of the nuclear factor CREB, we assayed the activity of putative CREB kinases (PKA: Yamamoto et al., 1988; CaMK: Sheng et al., 1990; PKC: Yamamoto et al., 1988 and de Groot et al., 1993; RSK: Böhm et al., 1995 and Xing et al., 1996; $p70^{S6K}$: de Groot et al., 1994) in OP cells treated with kainate, carbachol, and growth factors.

First, we measured CaMK II autophosphorylation, which has been shown to accompany enzyme activation in different systems (McNicol et al., 1990; Bading et al., 1993). Figure 5*A* shows that kainate and carbachol stimulated ^{32}P incorporation into CaMK II. The effect of kainate was rapid and transient, reaching a maximum within 2 min (3.2-fold increase; $n = 5$). In contrast, stimulation with TPA and growth factors did not lead to CaMK activation (Fig. 5*A*, and data not shown).

To analyze PKC activation, we measured the *in vivo* phosphorylation of two well characterized PKC-specific substrates: the myristoylated alanine-rich C kinase substrate MARCKS (Aderem, 1992) and the growth-associated protein GAP-43 (Skene, 1989). Both proteins were found to be phosphorylated shortly after stimulation with kainate, carbachol, and the PKC activator TPA (Fig. 5*B*, and data not shown). In particular, kainate caused a 2.9-fold stimulation of PKC activity (measured as MARCKS phosphorylation; $n = 2$) within 2 min of incubation. Exposure to the combination of PDGF and bFGF produced only a moderate and steady increase in MARCKS phosphorylation (Fig. 5*B*). Kainate and carbachol, but not growth factors, also induced PKC translocation to the membrane, as assayed by binding of radiolabeled phorbol esters to cultured OP cells (data not shown). These data indicate further that stimulation of glutamate and acetylcholine receptors in OP cells results in PKC activation.

We next examined the activation of RSK that has been proposed recently to mediate CREB phosphorylation in response to mitogenic signals (Böhm et al., 1995; Xing et al., 1996). Because RSK is a direct effector of the Ras/MAPK cascade (Blenis, 1993), we analyzed activation of this pathway in OP cells at three distinct

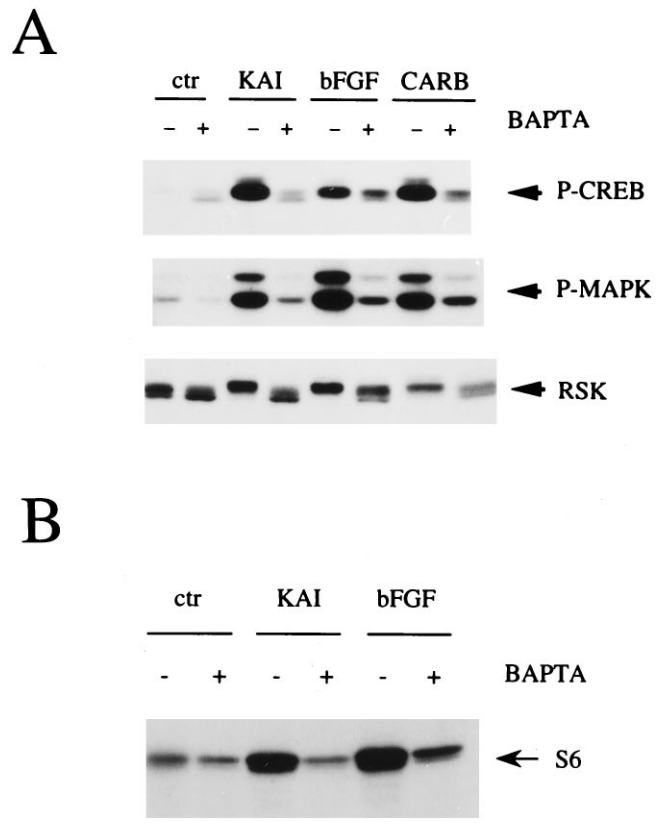

Figure 4. The effects of kainate, carbachol, and growth factors on CREB and on the MAPK/RSK pathway require intracellular Ca^{2+}. *A*, OP cells were preincubated with DMSO (− *lanes*) or 60 μM BAPTA-AM (+ *lanes*) for 2 hr. Cells were then treated with the stimulating agents as indicated (*KAI*: 300 μM kainate for 5 min; *CARB*: 300 μM carbachol for 5 min; *bFGF*: 10 ng/ml bFGF for 15 min). Aliquots of the same cell extracts were sequentially immunoblotted with anti-P-CREB, anti-P-MAPK, and anti-RSK antisera. The 43 kDa CREB phosphoprotein (*P-CREB*), the 44 and 42 kDa ERK-1 and ERK-2 phosphoproteins (*P-MAPK*), and the *RSK* protein are indicated by the *arrows*. Note the mobility shift of RSK (kainate, carbachol, and bFGF lanes in the absence of BAPTA), attributable to hyperphosphorylation. *B*, Effect of BAPTA on kainate- and bFGF-induced RSK activity. OP cells were preincubated with DMSO (− *lanes*) or 45 μM BAPTA-AM (+ *lanes*) for 90 min. Cells then were treated with the stimulating agents as indicated (*KAI*: 300 μM kainate for 5 min; *bFGF*: 10 ng/ml bFGF for 15 min). Cell extracts were immunoprecipitated with anti-RSK antiserum, and RSK activity in the immune complex was determined using GST-S6 fusion protein as a substrate. Incorporation of phosphate into S6 was detected by autoradiography after SDS-PAGE analysis. GST-S6 phosphoprotein is indicated by the *arrow*.

levels: (1) GTP binding to Ras, (2) dual phosphorylation of MAP kinases ERK-1 and ERK-2, and (3) RSK phosphorylation and kinase activity. The results in Figure 6*A* indicate that the proportion of GTP-bound, active Ras was increased significantly only by treatment with the growth factors PDGF and bFGF, and not by kainate, carbachol, or TPA. When MAPK tyrosine phosphorylation was assessed by immunoblot analysis with phospho-specific antibodies, however, all of these signals appeared to stimulate ERK-1 and ERK-2 (Fig. 4*B*, and data not shown). The effects of kainate and carbachol on MAPK were more transient than those of growth factors and required influx of extracellular Ca^{2+} (Fig. 6*B*, and data not shown).

RSK activity in stimulated OP cells was assayed by immunoprecipitation with anti-RSK antibody, combined to *in vitro* kinase assays, using S6 protein as a substrate. Kainate, carbachol, TPA, and growth factors significantly stimulated RSK activity (Fig. 6*C*, and data not shown). In support of these functional data, we also observed a reduction of RSK electrophoretic mobility in cells treated with these stimulating agents, as detected by immunoblot analysis with anti-RSK antibodies (Fig. 6*D*, and data not shown). These slower migrating bands represent hyperphosphorylated forms of RSK, which are likely to be catalytically active (Vik et al., 1990). Incubation with BAPTA-AM clearly reduced MAPK and RSK activation in resting cells, as well as in cells stimulated with kainate, carbachol, and bFGF (Fig. 4*A*), indicating that the activity of the MAPK/RSK pathway in OP cells is dependent on intracellular Ca^{2+}.

We next examined activation of $p70^{S6K}$ by immune complex-S6 protein kinase assays and immunoblot analysis in OP cells. Kainate caused only a slight retardation in the electrophoretic mobility of $p70^{S6K}$, without any detectable change in kinase activity (data not shown), indicating that kainate-stimulated $p70^{S6K}$ phosphorylation is not sufficient to activate the enzyme. In contrast, growth factors stimulated both $p70^{S6K}$ phosphorylation and activation (data not shown).

The involvement of PKA in kainate-, carbachol-, and growth factor-induced CREB phosphorylation was ruled out on the basis of two distinct observations. First, none of these agonists significantly increased cAMP levels in OP cells (data not shown). Second, treatment with forskolin (50 μM), which caused an 11-fold increase in cAMP levels (data not shown) and likely full activation of PKA, resulted in a weaker induction of CREB phosphorylation as compared with kainate, carbachol, and growth factors (Fig. 1).

In conclusion, our biochemical screening for inducible kinase activity indicates that in OP cells CaMK and PKC are preferentially stimulated by Ca^{2+} influx, and $p70^{S6K}$ exclusively by growth factors, whereas RSK is the only potential CREB kinase whose activity is substantially enhanced by both types of signals.

Specific block of the MAPK pathway inhibits CREB phosphorylation induced by calcium influx, growth factors, and TPA

To elucidate the individual contribution of intracellular pathways in signaling to CREB, various kinase inhibitors were tested for their potency and specificity on the distinct intracellular pathways described above. PD 098059 has recently been characterized as a selective inhibitor of the MAPK pathway (Alessi et al., 1995; Dudley et al., 1995). This compound was found to specifically inhibit MEK, the protein kinase that phosphorylates and activates MAP kinase (Alessi et al., 1995). To test whether PD 098059 was also effective in our system, OP cells were incubated for 1 hr with PD 098059 before stimulation, and then MAPK and RSK phosphorylation were assayed in cell extracts by immunoblot analysis. PD 098059 inhibited basal as well as kainate-, TPA-, and bFGF-induced MAPK phosphorylation (Fig. 7*A,B*). In particular, 50 μM PD 098059 completely suppressed MAPK phosphorylation by kainate but was less effective in counteracting the effects of bFGF and TPA, which are stronger activators of the MAPK pathway. This differential potency of the MEK inhibitor is likely to depend on the strength of the stimulus, as observed previously in other cellular systems (Alessi et al., 1995). In all of the conditions studied, RSK phosphorylation always paralleled MAPK phosphorylation, consistent with the role of RSK as a downstream effector of the MAPK pathway (Fig. 7*A,B*). As expected, PD 098059 also inhibited the RSK phosphotransferase activity induced by kainate and bFGF (Fig. 7*C*).

In the presence of 50 μM PD 098059, CREB phosphorylation in

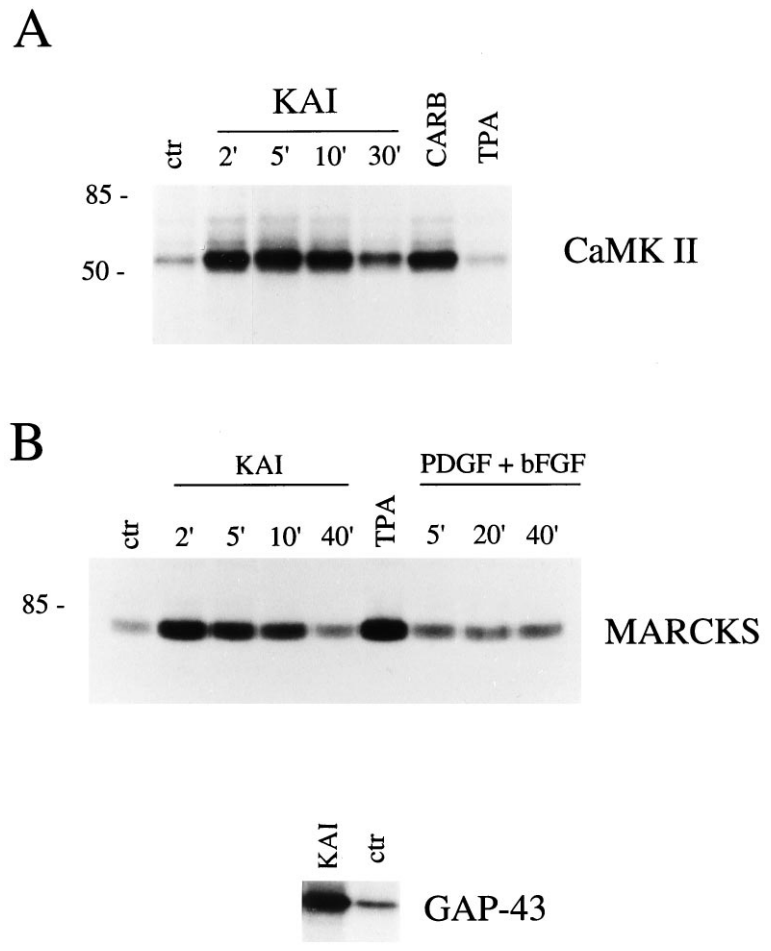

Figure 5. CaMK II and PKC activation in response to kainate and carbachol. *A*, CaMK II autophosphorylation. OP cells were metabolically labeled with [^{32}P]-orthophosphate before stimulation and treated as indicated (*KAI*: 300 μM kainate for 2 min unless otherwise indicated; *CARB*: 300 μM carbachol for 2 min; *TPA*: 100 nM TPA for 5 min), lysed, and immunoprecipitated with α- and β-subunit-specific anti-CaMK II antibodies. Incorporation of phosphate into CaMK II was detected by autoradiography after SDS-PAGE analysis. *B*, *In vivo* phosphorylation of PKC substrates. OP cells were metabolically labeled with [^{32}P]-orthophosphate and treated as indicated (*KAI*: 300 μM kainate for various periods of time; *TPA*: 100 nM TPA for 5 min; *PDGF + bFGF*: 10 ng/ml PDGF + 10 ng/ml bFGF for various periods of time), lysed, and immunoprecipitated with anti-MARCKS or anti-GAP-43 antisera, followed by SDS-PAGE analysis. Incorporation of phosphate into MARCKS and GAP-43 was detected by autoradiography. The 80 kDa MARCKS and the 43 kDA GAP-43 phosphoproteins are indicated.

kainate-, TPA-, and bFGF-treated cells was reduced by ~70% (Fig. 7*A*), suggesting that the MAPK pathway mediates, at least in part, CREB regulation by Ca^{2+} influx, PKC activation, and growth factors, respectively. Treatment with the MEK inhibitor did not affect CREB phosphorylation in unstimulated cells or in forskolin-treated cells (Fig. 7), indicating that PD 098059 did not interfere with basal and PKA-mediated regulation of CREB. Moreover, 50 μM PD 098059 did not inhibit the phosphorylation of CaMK by kainate (data not shown), demonstrating that its effects on kainate-stimulated CREB phosphorylation were not attributable to a nonspecific inhibition of the CaMK pathway.

Incubation of OP cells with higher concentrations of PD 098059 (100 μM) did not lead to a complete inhibition of CREB activation by any of the stimulating agents (Fig. 7*B*) (71% inhibition of kainate-induced CREB phosphorylation, $n = 4$; 87% inhibition of bFGF-induced CREB phosphorylation, $n = 3$). The residual bFGF-induced CREB phosphorylation observed in the presence of the MEK inhibitor is likely to be attributable to the incomplete inhibition of the MAPK pathway. So far, we have no evidence that additional pathways are involved in mediating the effect of growth factors on CREB phosphorylation. In fact, it is unlikely that the stimulation of the $p70^{S6K}$ pathway by bFGF has any role in CREB regulation, because rapamycin, an inhibitor of $p70^{S6K}$ activation (Chung et al., 1992), suppressed $p70^{S6K}$ phosphorylation in OP cells without affecting CREB Ser-133 phosphorylation (data not shown).

It is likely that in kainate-treated cells, in which high concentrations of PD 098059 decreased MAPK phosphorylation to undetectable levels (Fig. 7*B*), additional Ca^{2+}-activated pathways might contribute to the regulation of CREB phosphorylation. To determine whether activation of CaMK participates in CREB phosphorylation by kainate, we stimulated OP cells in the presence of KN-93, a CaMK inhibitor related to KN-62 (Sumi et al., 1991), which has been used successfully to establish a role for CaMK as mediator of nuclear events in several systems (Bading et al., 1993; Enslen and Soderling, 1994; Deisseroth et al., 1996). Preincubation of the cells with KN-93 significantly inhibited basal and kainate-induced autophosphorylation of CaMK (data not shown), demonstrating that in our conditions KN-93 was effective in blocking the activity of the enzyme. However, when KN-93 was tested for its ability to prevent CREB activation, the CaMK inhibitor caused only a moderate reduction (12% inhibition, $n = 6$) in the levels of phosphorylated CREB in kainate-treated cells (Fig. 7*B*). Induction of CREB phosphorylation by bFGF was not affected by the CaMK inhibitor (Fig. 7*B*), consistent with our findings that CaMK was not activated by growth factors in these cells.

Role of PKC

Finally, we examined the role of the Ca^{2+}-dependent conventional PKC isozymes (cPKC) on the induction of CREB phosphorylation by the various stimulating agents. Long-term treatment with TPA strongly decreased the levels of cPKC isoforms (Fig. 8*A*) and caused a complete inhibition of CREB phosphorylation by TPA and a partial inhibition of CREB phosphorylation by kainate and carbachol (Fig. 8*C*). Cells in which cPKC was

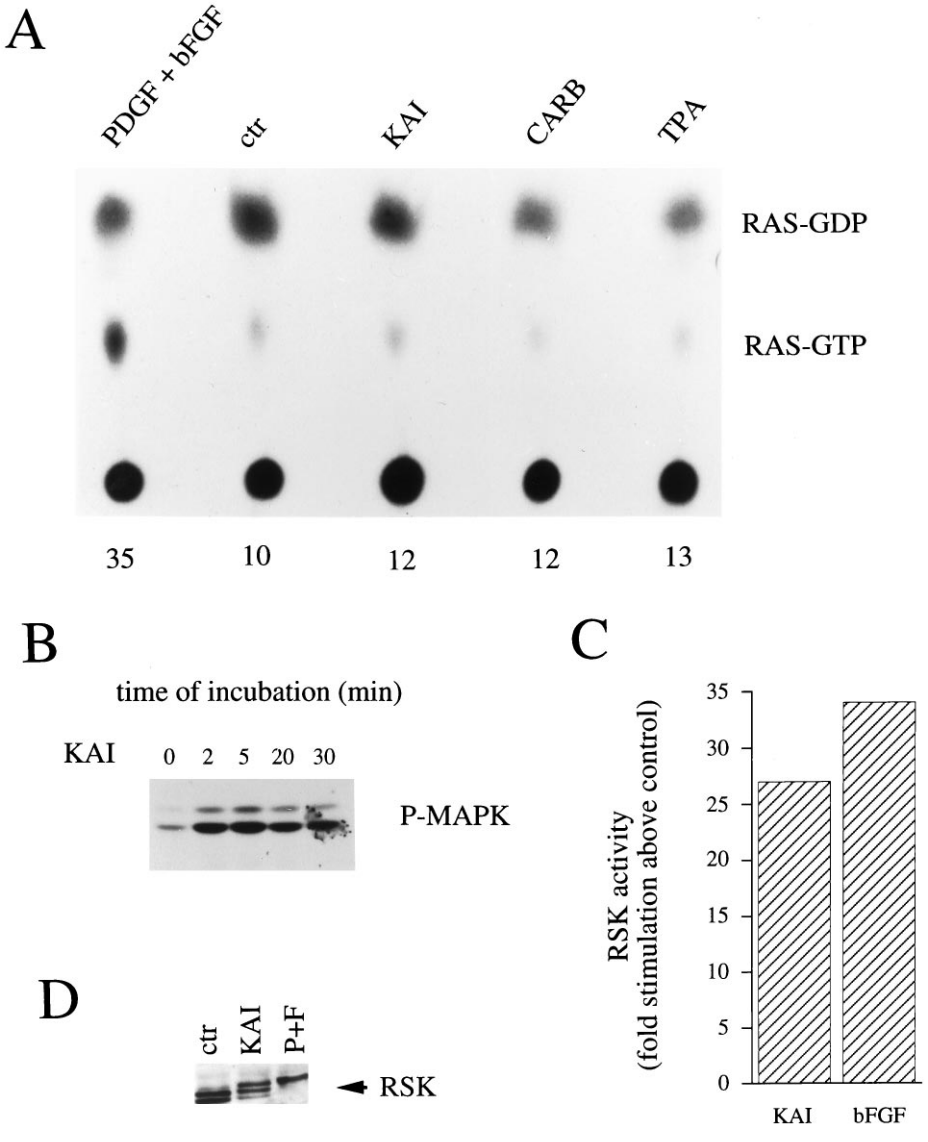

Figure 6. Activation of Ras, MAPK, and RSK by extracellular signals in OP cells. *A*, GDP/GTP binding to Ras in OP cells treated with vehicle (*ctr*), 300 μM kainate for 2 min (*KAI*), 300 μM carbachol for 2 min (*CARB*), 100 nM *TPA* for 5 min, 10 ng/ml PDGF + 10 ng/ml bFGF (*PDGF + bFGF*) for 5 min. Cells were metabolically labeled with [^{32}P]-orthophosphate before stimulation, lysed, and immunoprecipitated with anti-Ras antibodies. Guanine nucleotides bound to Ras were eluted and separated by thin layer chromatography. Position of GDP and GTP standards is shown. Content of GDP and GTP bound to Ras was quantified by phosphorimager analysis, and the percentage of GTP is indicated at the *bottom*. *B*, Time course of MAPK phosphorylation in response to kainate. Immunoblot analysis with an anti-P-MAPK antiserum of extracts from OP cells incubated for the indicated periods of time with 300 μM kainate. The 44 and 42 kDa ERK-1 and ERK-2 phosphoproteins (*P-MAPK*) are indicated. To allow a direct comparison of the kinetics of CREB and MAPK phosphorylation in response to kainate, the immunoblot analysis shown in Figures 1 and 6*B* was performed on the same cell extracts. Despite some quantitative variability among experiments, the average increase of MAPK phosphorylation after a 5 min stimulation with kainate was 80% of the increase induced by bFGF ($n = 5$). *C*, Increase of RSK activity in response to kainate and growth factors. OP cells were stimulated with 300 μM kainate for 5 min or with 10 ng/ml bFGF for 15 min. RSK activity was determined by immune complex kinase assay using S6 ribosomal protein as substrate. Data presented in the histogram are from one representative experiment and were confirmed in three other independent experiments. *D*, RSK mobility shift in response to kainate and growth factors. Cells were stimulated as indicated (*KAI*: 300 μM kainate for 5 min; *P+F*: 10 ng/ml bFGF + 10 ng/ml PDGF for 15 min). Immunoblot analysis of RSK was performed using an antiserum directed against the C-terminal peptide of RSK, which recognizes both the phosphorylated (slower migrating) and unphosphorylated (faster migrating) forms of the protein. The ~85 kDa RSK protein is indicated by the *arrow*.

downregulated also showed a reduced activation of MAPK in response to kainate, carbachol, and TPA (Fig. 8*B*, and data not shown), raising the possibility that in OP cells cPKC functions as an upstream regulator of the MAPK/RSK pathway, which in turn leads to CREB Ser-133 phosphorylation. In contrast, downregulation of cPKC caused a moderate reduction in the levels of phosphorylated MAPK on growth factor stimulation (Fig. 8*B*) and did not significantly affect the induction of CREB phosphorylation by bFGF and PDGF (Fig. 8*C*) (long-term treatment with TPA partially inhibited growth factor-induced CREB phosphorylation in only one of five experiments). We cannot exclude at present the possibility that in OP cells, TPA-insensitive atypical PKC isoforms may be involved in the transduction of growth factor signals to CREB.

DISCUSSION

We have characterized the molecular events leading to regulation of the transcription factor CREB in a homogeneous population of primary neural cells, highlighting a central role for the MAPK pathway as an intermediary between cell surface receptors and intracellular Ca^{2+} and CREB phosphorylation. We have shown that in cortical OPs the MAPK pathway is activated in a Ca^{2+}-dependent fashion on stimulation of glutamate receptor channels and G-protein-coupled cholinergic receptors, as well as in response to growth factors. The MAPK pathway therefore can integrate these distinct upstream signals and transduce them to the nucleus, leading to the phosphorylation of CREB at Ser-133, an event necessary for its transcription-activating function.

The mechanism of MAPK activation is likely to be different for the distinct receptor systems analyzed in our study. The signal transduction pathway linking tyrosine kinase receptors with MAPK activation has been studied extensively in many cell types (Marshall, 1995) and includes ligand binding, receptor dimerization and autophosphorylation, and recruitment of Grb2/Sos complexes that activate Ras by inducing its association with GTP. Raf kinases bind Ras · GTP and are activated by several phosphorylation events, which may involve protein kinases such as PKCα, Src, and KSR (Kolch et al., 1993; Downward, 1995; Marais et al., 1995). Raf kinase activation is then followed by sequential phosphorylation and activation of MEK, MAPK, and RSK. Our analysis shows that such a mechanism is likely to operate also in OP

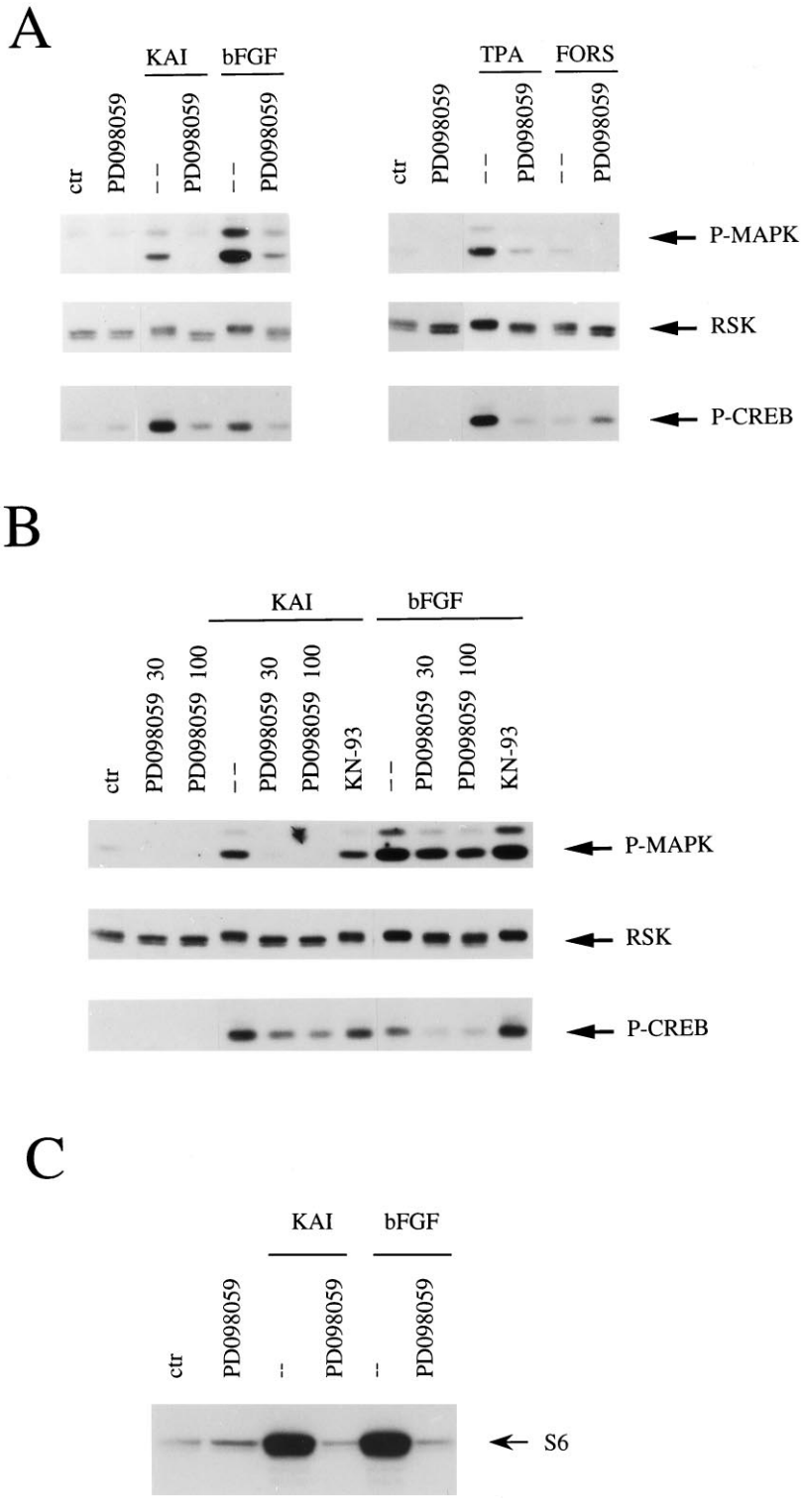

Figure 7. Effect of PD 098059 and KN-93 on MAPK, RSK, and CREB phosphorylation. *A*, OP cells were preincubated with either DMSO or 50 μM PD 098059 (*PD098059 lanes*) for 1 hr. Cells were then stimulated with the indicated agonists (*KAI*: 300 μM kainate for 5 min; *bFGF*: 10 ng/ml bFGF for 15 min; *TPA*: 100 nM TPA for 10 min; *FORS*: 50 μM forskolin for 10 min). *B*, OP cells were preincubated with DMSO, 30–100 μM PD 098059, or 20 μM KN-93 for 1 hr, as indicated. Cells were then stimulated with the indicated agonists (*KAI*: 300 μM kainate for 5 min; *bFGF*: 10 ng/ml bFGF for 15 min). Aliquots of the same cell extracts were sequentially immunoblotted with anti-P-MAPK, anti-RSK, or anti-P-CREB antiserum. The 43 kDa CREB phosphoprotein (*P-CREB*), the 44 and 42 kDa ERK-1 and ERK-2 phosphoproteins (*P-MAPK*), and the ~85 kDa *RSK* protein are indicated by the *arrows*. *C*, Effect of PD 098059 on kainate- and bFGF-induced RSK activity. OP cells were preincubated with DMSO or 75 μM PD 098059 (*PD098059 lanes*) for 1 hr. Cells were then treated with the stimulating agents as indicated (*KAI*: 300 μM kainate for 5 min; *bFGF*: 10 ng/ml bFGF for 15 min). Cell extracts were immunoprecipitated with anti-RSK antiserum, and RSK activity in the immune complex was determined using GST-S6 fusion protein as a substrate. Incorporation of phosphate into S6 was detected by autoradiography after SDS-PAGE analysis. GST-S6 phosphoprotein is indicated by the *arrow*.

cells stimulated with bFGF and PDGF, because these growth factors substantially increase the proportion of Ras bound to GTP in these cells (Fig. 6*A*).

Kainate- and carbachol-induced MAPK activation is essentially triggered by the transmembrane influx of Ca^{2+} caused by glutamatergic and cholinergic receptor stimulation. Recent studies in PC12 cells and in hippocampal neurons have demonstrated that similar to growth factors, Ca^{2+} influx can also signal to MAPK through the activation of Ras (Rosen et al., 1994; Farnsworth et al., 1995; Lev et al., 1995; Rusanescu et al., 1995; Rosen and Greenberg, 1996; for review, see Finkbeiner and Greenberg, 1996); however, we do not observe any increase in GTP binding to Ras after Ca^{2+} influx in OP cells (Fig. 6*A*). It is possible that on treatment with kainate and carbachol, basal levels of Ras · GTP are sufficient for activation of the MAPK pathway by upstream regulatory elements. Alternatively, in OPs the signal transduction pathway linking Ca^{2+}-permeable membrane channels with MAPK may not include Ras. PKCα has been shown to phosphor-

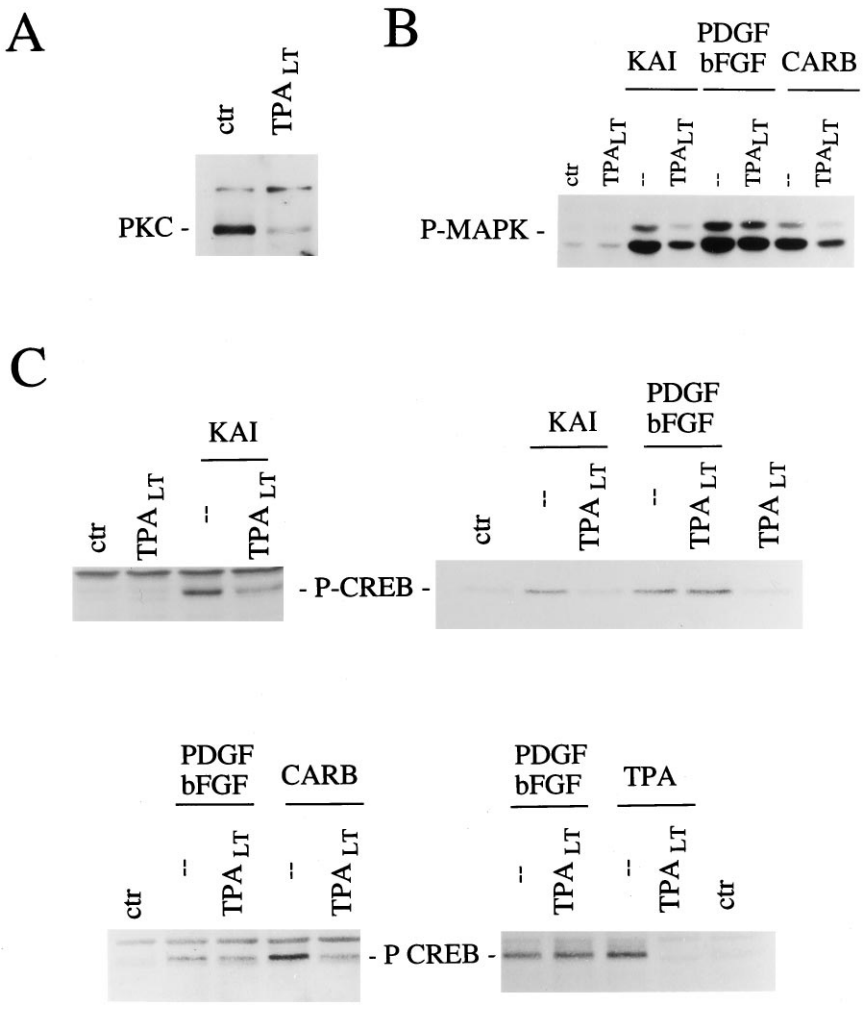

Figure 8. cPKC downregulation inhibits CREB activation by kainate, carbachol, and TPA, without affecting stimulation by growth factors. *A*, Immunoblot analysis with an anti-Pan-PKC antiserum of extracts from OP cells incubated for 10 hr with 0.1% DMSO (*ctr*) or 8 nM TPA (TPA_{LT}). The 80 kDa PKC isozyme is indicated. *B* and *C*, OP cells were preincubated with DMSO or 8 nM TPA for 10 hr (TPA_{LT}), as indicated. Cells were then stimulated with the indicated agonists (*KAI*: 300 μM kainate for 5 min; *CARB*: 300 μM carbachol for 5 min; *TPA*: 100 nM TPA for 10 min; 10 ng/ml *PDGF* and *bFGF* for 15 min). Cell extracts were immunoblotted with anti-P-MAPK (*B*) or anti-P-CREB (*C*) antiserum. The 43 kDa CREB phosphoprotein and the 44 and the 42 kDa ERK-1 and ERK-2 phosphoproteins (*P-MAPK*) are indicated.

ylate and activate Raf *in vitro* and *in vivo* through a mechanism that may parallel Raf regulation by Ras and Src (Kolch et al., 1993). Interestingly, we have demonstrated that in OP cells transmembrane Ca^{2+} influx is sufficient to translocate PKC to the membrane and to stimulate its catalytic activity (Fig. 5*B*, and data not shown). Moreover, the phorbol ester TPA mimics the effect of kainate and carbachol on PKC stimulation (Fig. 5*B*) and leads to MAPK activation without activating Ras (Fig. 6). Finally, downregulation of an 80 kDa PKC isozyme inhibits MAPK activation by Ca^{2+} influx (Fig. 8*B*). Taken together, these observations suggest that PKC may integrate Ca^{2+} signals in OP cells to activate the MAPK pathway. Additional studies are needed to elucidate whether Ras is involved in MAPK activation by Ca^{2+} influx in OP cells.

A striking observation in our studies is that the activity of the MAPK pathway is influenced strongly by intracellular Ca^{2+} levels. Chelation of cytoplasmic Ca^{2+} by BAPTA inhibits kainate-, carbachol-, and growth factor-induced MAPK and RSK activation (Fig. 4). Although an inhibitory effect of BAPTA on the signal transduction initiated by kainate and carbachol is consistent with its dependence on external Ca^{2+} (see discussion above), it is surprising that the growth factors also depend on Ca^{2+} to activate the MAPK pathway. In our culture conditions, bFGF, alone or in combination with PDGF, causes a moderate rise of $[Ca^{2+}]_i$ in only 22% of the OP cells (Fig. 2). This small Ca^{2+} response is unlikely to account for the strong stimulation of the MAPK pathway by tyrosine kinase signals (for example, see Figs. 4, 6). It is possible, however, that resting levels of Ca^{2+} are essential as a co-factor for the function of some regulatory elements of the MAPK pathway. This hypothesis is supported by the findings that chelation of cytoplasmic Ca^{2+} by BAPTA also affects the basal phosphorylation of MAPK and RSK (Fig. 4). Although the Ca^{2+}-dependent step in this phosphorylation cascade has not yet been identified, recent studies have indicated a similar Ca^{2+}-requirement for the function of the MAPK pathway (Burgering et al., 1993; Böhm et al., 1995).

In many cellular systems, activation of the MAPK pathway by growth factors has been implicated in the regulation of gene transcription (Treisman, 1996) and has been associated with the cellular responses of proliferation, differentiation, and transformation (Marshall, 1995). Ca^{2+}-induced activation of MAPK might result in similar biological effects, but only a few studies have analyzed the physiological role of this pathway. Rusanescu et al. (1995), using dominant negative forms of Src and Ras, were able to show that both oncoproteins (probably acting through the MAPK pathway) were necessary to mediate the induction of *NGFI-A* expression and neurite outgrowth by Ca^{2+} signals in PC12 cells. These findings demonstrate that Ca^{2+} and growth factor signals may converge to identical effectors and trigger similar biological processes. In our study in OP cells, we have presented several lines of evidence implicating the MAPK/RSK pathway in the regulation of the transcription factor CREB by

both Ca^{2+} influx and growth factors. First, the kinetics of CREB phosphorylation by the two types of signals parallel the kinetics of MAPK phosphorylation (Figs. 1 and 6, and data not shown). Second, CREB, MAPK, and RSK phosphorylation display the same dependence on intracellular Ca^{2+} (Fig. 4). Third, downregulation of PKC inhibits both MAPK and CREB phosphorylation triggered by Ca^{2+} influx (Fig. 8). Finally, selective inhibition of the MAPK/RSK pathway by PD 098059 reduces Ca^{2+}- and growth factor-induced CREB phosphorylation (Fig. 7).

RSK has been shown to phosphorylate CREB Ser-133 *in vitro* and *in vivo* on growth factor stimulation (Ginty et al., 1994; Böhm et al., 1995; Xing et al., 1996). Therefore, this enzyme is an excellent candidate for catalyzing the reaction in OP cells; however, other MAPK-activated CREB kinases may also exist in OP cells. Böhm et al. (1995) have reported that some kinases other than RSK were activated by growth factors in melanocytes and displayed CREB kinase activity *in vitro*.

Initially described as a transcription factor activated by stimuli that raise intracellular levels of cAMP and lead to PKA activation (Gonzalez and Montminy, 1989), CREB was found subsequently to be phosphorylated also at Ser-133 on Ca^{2+} influx or growth factor stimulation (Sheng et al., 1990; Ginty et al., 1994). Therefore, CREB seems to act as an element of convergence and cross-talk between distinct signaling pathways, rather than as a target of one single pathway. Studies on Ca^{2+} signal transduction in PC12 cells and hippocampal neurons have proposed that CaM kinases may be the Ca^{2+}-activated enzymes that phosphorylate CREB on membrane depolarization (Sheng et al., 1991; Deisseroth et al., 1996). Our results in OP cells indicate that CaM kinases are involved in the transduction of Ca^{2+} signals to the nucleus to a lesser extent than the MAPK pathway (Fig. 7). Such differential contribution of the Ca^{2+} signaling pathways to CREB phosphorylation may be attributable to the different neural cell types analyzed in these studies. On the other hand, it should also be noted that in the cellular systems considered previously, voltage-dependent Ca^{2+} channels (Sheng et al., 1991) and NMDA receptors (Deisseroth et al., 1996) were the major source of Ca^{2+} entry, whereas in OP cells stimulated with kainate and carbachol, Ca^{2+} flows into the cells through different channels, i.e., mainly non-NMDA receptors and store-operated channels. It is therefore possible that the route of Ca^{2+} entry affects which pathways propagate Ca^{2+} signals to the nucleus.

CREB phosphorylation at Ser-133 is usually followed by transcriptional activation of CRE-dependent genes (Gonzalez and Montminy, 1989; Sheng et al., 1991; Ginty et al., 1994; Xing et al., 1996). The mechanism underlying this process involves the binding of P-Ser-133 CREB to a CREB binding protein (CBP) (Chrivia et al., 1993), followed by interaction of this complex with the basal transcriptional machinery; however, the transactivation potential of CREB may be controlled by some additional events (Sun et al., 1994; Nakajima et al., 1996). The complexity of CREB regulation has been emphasized recently by two separate studies, which are particularly relevant for our analysis. Xing et al. (1996) reported that RSK2, a member of the RSK family of protein kinases, promoted CREB activation by phosphorylating the Ser-133 residue. On the other hand, Nakajima et al. (1996) proposed that RSK might interfere negatively with the CREB-mediated transactivation by inhibiting CBP function. Clearly, additional studies are needed to clarify the physiological role of the MAPK/RSK pathway on the regulation of CRE-dependent transcription on growth factor stimulation as well as Ca^{2+} influx.

Oligodendrocyte development is tightly regulated by cAMP levels (McMorris et al., 1990), growth factors (Barres and Raff, 1994), and ion fluxes (Gallo et al., 1996). Our results identify molecular mechanisms that in OP cells can convey the information of these distinct signals to nuclear factors. These studies therefore may provide the basis for understanding how different environmental signals influence developmental progression of oligodendroglial cells.

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