Neuronal Calcium Activates a Rap1 and B-Raf Signaling Pathway via the Cyclic Adenosine Monophosphate-dependent Protein Kinase*

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Savraj S. Grewal‡§, Angela M. Horgan‡, Randall D. York‡, Ginger S. Withers§, Gary A. Banker‡, and Philip J. S. Stork‡¶

From the ‡Vollum Institute, L474, the §Department of Cell and Developmental Biology, and the ¶Center for Research on Occupational and Environmental Toxicology, Oregon Health Sciences University, Portland, Oregon 97201

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

Activity-dependent changes in neuronal processes such as synaptic plasticity and neuronal survival are mediated in large part through elevations in intracellular calcium levels (1–3). In many cases, this involves stimulation of calcium influx, particularly via voltage-operated L-type channels or receptor-operated N-methyl-D-aspartate channels (1, 4). Many of the downstream functional consequences of calcium influx involve the regulation of gene transcription (5–7). As such, numerous studies have begun to examine signal transduction pathways that link neuronal calcium to the regulation of transcription factor activity. These studies have revealed a complexity of calcium-dependent kinase signaling cascades (8).

The mitogen-activated protein/extracellular signal-regulated kinases (MAP kinases or ERKs) are emerging as important targets for neuronal calcium signaling. ERK activation by neuronal calcium influx has been demonstrated both in vitro, using isolated neuronal cultures and the PC12 cell line (9–12), and in vivo after stimulation of neuronal activity (13–15). Changes in synaptic plasticity are also associated with increases in ERK activity (16, 17). In addition, a role for ERK activity has been demonstrated for both hippocampal long term potentiation (18–20) and cerebellar long term depression (21). These actions may underlie a requirement for ERKs in learning and memory (22–25).

A major issue concerns the mechanism by which calcium influx stimulates ERK activity. ERKs have been best examined in the context of growth factor signaling via receptor tyrosine kinases (26). These studies have identified a ubiquitous ERK cascade involving the stimulation of the small G-protein, Ras, and the kinases, Raf-1 and MEK (26). Since both growth factors and neuronal calcium often exhibit synergistic actions, it is possible that they may share common signaling pathways. Thus, in both in vitro neuronal cultures and PC12 cells, it has been demonstrated that depolarization-mediated opening of L-type calcium channels can activate Ras (9, 27). The mechanism by which this occurs is unclear, but it may involve Shc phosphorylation, perhaps via transactivation of the epidermal growth factor receptor (28, 29), stimulation of the tyrosine kinase Src (10) or Pyk2 (30), or activation of the calmodulin-sensitive Ras guanine nucleotide exchange factor (GEF), Ras-GRF (27). Moreover, recent studies have also identified a novel Ras GEF that can be activated directly by calcium and that may contribute to neuronal calcium stimulation of ERKs (31, 32).

Although Ras represents an important target for neuronal calcium activation of ERKs, it is possible that calcium influx may additionally signal via alternate routes that are Ras-independent. We have recently identified a novel pathway regulating ERK activation via nerve growth factor (NGF) and cAMP (33–35). Unlike the well studied Ras-dependent ERK cascade, this pathway involves the PKA-dependent stimulation of the Ras-related small G-protein, Rap1, and the downstream kinase, B-Raf (33). Given the ability of neuronal calcium to increase intracellular cAMP levels and PKA activity via calmi-

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† To whom correspondence should be addressed: The Vollum Institute, Oregon Health Sciences University, L474, S.W. Sam Jackson Park Road, Portland, OR 97201. Tel.: 503-494-5494; Fax: 503-494-4976; E-mail: stork@ohsu.edu.
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odulin-sensitive adenylyl cyclases (36), this pathway may represent an additional target for activity-dependent signaling. Indeed, studies in Aplysia have shown that long term facilitation involves PKA activation of ERKs, consistent with a role for Rap1/B-Raf (16). In addition, the identification of two novel families of Rap1 GEFs that can activated by direct binding to calcium and cAMP, respectively, also suggest a role for Rap1 in neuronal calcium signaling (37, 38).

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

EXPERIMENTAL PROCEDURES

Materials—PC12-G5 cells were kindly provided by R. Nishi (Oregon Health Sciences University, Portland, OR). Agarose-conjugated antibodies to ERK1 and ERK2 (ε-16), antibodies to B-Raf and Raf-1, and recombinant MEK-1 protein were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Flag M2 antibody was purchased from Sigma. Forskolin, KT5720, and N-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide (H89), N-[4-aminobutyl]-1-naphthalenesulfonamide, HCl (W12), and N-[4-aminobuty]-1-naphthalenesulfonamide, HCl (W13) were purchased from Calbiochem.

Phosphorylation-specific ERK antibodies that recognize phosphorylated ERK1 and ERK2 at residues threonine 183 and tyrosine 185 were purchased from Sigma. Forskolin, KT5720, and N-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide (H89), N-[4-aminobutyl]-1-naphthalenesulfonamide, HCl (W12), and N-[4-aminobuty]-1-naphthalenesulfonamide, HCl (W13) were purchased from Calbiochem.

All other reagents were from Sigma.

Cell Culture—PC12 cells were maintained in Dulbecco’s modified Eagle’s medium plus 0.5% horse serum for 16 h at 37 °C in 5% CO2 prior to treatment with various reagents. The cells were then starved overnight, and the supernatant was collected. ERK2 was immunoprecipitated from lysates containing equal protein amounts per treatment condition, and kinase activity was measured by immune complex assay as described (33) using [γ-32P]ATP with myelin basic protein as substrate. For Raf assays, untreated and treated cells were lysed in 1% Nonidet P-40 containing 10 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. The lysates were spun at low speed to remove nuclei, and the supernatant was collected. Raf-1 or B-Raf was immunoprecipitated from equal protein amounts of lysate per treatment condition, and activity was measured by an immune complex assay as described (33) using [γ-32P]ATP with MEK as substrate. The reaction products of all kinase assays were resolved by 10% SDS-polyacrylamide gel and analyzed with a Phosphoimager (Molecular Dynamics, Inc., Sunnyvale, CA).

RESULTS

Neuronal Depolarization Activates ERKs in a PKA-dependent Manner—Membrane depolarization of PC12 cells following the addition of KCl activated ERK2 in a concentration-dependent manner. (Fig. 1A). Sixty millimolar KCl induced a rapid and sustained increase in ERK activity as assessed by both immune complex kinase assay (Fig. 1B) and phosho-specific antibodies (Fig. 1C), with maximal increases occurring at 5–10 min. This increase in ERK activity was abolished by pretreatment with the L-type calcium channel antagonist, nifedipine (5 μM), consistent with a role for calcium influx (Fig. 2, A and B).

Interestingly, inhibition of PKA, using either H89 (40, 41) or KT5720 (42), also blocked KCl-induced increase in ERK activity (Fig. 2, A (upper panel), B, and C) and phosphorylation (Fig. 2A, lower panel). This requirement for PKA in the actions of depolarization is consistent with the possible stimulation of calcium/calmodulin-activated adenylyl cyclases. To address this, we also examined the requirement of calmodulin for the activation of ERKs by depolarization. Pretreatment with W13, a calmodulin antagonist (43), blocked the depolarization-mediated increase in ERK activation (Fig. 2D). In contrast, W12, a structurally related, inactive analog of W13 (43), had no effect. Finally, this PKA-dependent activation of ERKs appeared specific for depolarization; ERK activation by ionomycin, a calcium ionophore, was unaffected by pretreatment with H89 (Fig. 2E).

We further examined this requirement for PKA in the activation of ERKs in two other neuronal model systems. We first...
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**Fig. 1. Activation of ERKs following depolarization of PC12 cells.** Treated cells were harvested for either immune complex kinase assay using myelin basic protein as a substrate or Western blotting using phosphospecific ERK1/2 antibodies. A, PC12 cells were treated with KCl at the concentrations indicated for 10 min. Immune complex assays with equal protein amounts of cell lysate were performed. A representative autoradiogram with the position of myelin basic protein (MBP) is shown. B, PC12 cells were treated with KCl (60 mM) for the times indicated. Immune complex assays with equal protein amounts of cell lysate were performed. A representative autoradiogram is shown. C, PC12 cells were treated with KCl (60 mM) for the times indicated. Western blotting with equal protein amounts of cell lysate was performed. Upper panel, probe with phospho-specific ERK1/2 antibody (Phospho-ERK1/2). Bottom panel, parallel Western blot probed with ERK1/2 antibody (ERK1/2).

looked at PC12 cells that had been neuronally differentiated by culturing for 10 days in the presence of nerve growth factor. As with undifferentiated cells, depolarization of neuronal PC12 cells also activated ERKs in a PKA-dependent manner as assessed by H89 inhibition of ERK phosphorylation (Fig. 3A). We next examined the actions of depolarization in dissociated hippocampal neuron cultures. Using immunofluorescence, we found that depolarization of neurons for 10 min induced a marked increase in the phosphorylation of ERKs both within the soma and dendrites (Fig. 3, B and C). Interestingly, ERK phosphorylation appeared largely absent from axons (data not shown). Pretreatment with H89 inhibited this activation of ERKs (Fig. 3, B and C). This requirement for PKA in ERK phosphorylation within hippocampal neurons was further confirmed by Western blot (Fig. 3C iii). Taken together, these data suggest that the PKA-dependent activation of ERKs may be a common target for calcium influx signaling in neuronal systems.

Neuronal Depolarization Activates B-Raf, but Not Raf-1, via PKA—Previous studies have identified Raf-1 as the primary Raf isoform involved in signaling to ERKs, particularly with respect to growth factor actions in nonneuronal cells. In contrast, B-Raf is the major neuronal Raf isoform, being highly expressed within the CNS and PC12 cells. The involvement of these Raf isoforms in neuronal calcium-mediated signaling has not been well established. We therefore examined the contribution of these two isoforms to depolarization-induced calcium signaling. Membrane depolarization of PC12 cells activated B-Raf, an effect that was completely blocked by pretreatment with the PKA inhibitor H89 (Fig. 4A). In contrast, Raf-1 was not activated by depolarization, even in cells where depolarization induced a robust phosphorylation of ERKs (Fig. 4B).

Neuronal Depolarization Activates Rap1 via PKA and Induces Formation of a Rap1/B-Raf Signaling Complex—B-Raf can be activated by both Ras and Rap1. The mechanism by which this occurs involves the recruitment of B-Raf by either active Rap1 or Ras to the membrane, where it can then subse-

quently be stimulated. A requirement for PKA in the activation of ERKs suggests a possible role for Rap1. Thus, we next determined the relative contribution of Rap- and Ras-dependent pathways by first examining a requisite step in B-Raf activation: association of the kinase with its upstream small G-protein activator. Membrane depolarization of PC12 cells led to a strong association of Rap1 with B-Raf (Fig. 5A i). This effect was similar to that seen with RapV12, a constitutively activated mutant of Rap1. Furthermore, the B-Raf that associated with Rap1 was capable of phosphorylating MEK-1 (Fig. 5A ii). In contrast, while NGF led to a robust association of Ras with B-Raf, membrane depolarization had no effect (Fig. 5B i).

The data above suggest that calcium influx may target a Rap1-dependent signaling pathway. Therefore, we directly examined the activation of Rap1 by monitoring GTP loading. Depolarization of PC12 cells led to a marked increase in Rap1 GTP loading (Fig. 6A). This effect was similar in magnitude to that observed with both the activator of adenylate cyclase, forskolin, and the constitutively active form of Rap1, RapV12 (Fig. 6A). Moreover, the depolarization-induced stimulation of Rap1 was completely reversed by pretreatment with the PKA inhibitor H89 (Fig. 6B).

**DISCUSSION**

The present study describes a novel mechanism by which calcium influx may stimulate ERKs in neurons (Fig. 7). Depolarization of PC12 cells, which stimulates calcium influx via L-type calcium channels induced the binding and activation of B-Raf by Rap1. Moreover, the stimulation of both Rap1 and B-Raf occurred via PKA. The major downstream consequence of Rap1/B-Raf signaling is the stimulation of ERKs (33). We further demonstrated that activation of ERKs following depolarization-mediated calcium influx in both PC12 cells and hippocampal neurons occurred via a PKA-dependent pathway. This is consistent with the involvement of a Rap1/B-Raf signaling cascade. While previous studies have either shown or inferred a role for Ras/Raf signaling to ERKs in neurons, the present data identify the Rap1/B-Raf pathway as an important additional target for neuronal calcium signaling. Both proteins are highly expressed in the CNS, possibly at postsynaptic sites. Additionally, B-Raf expression is specifically up-regulated during long term potentiation (44). As such, the Rap1/B-Raf pathway may be an important regulator of activity-dependent neuronal function.

Although a requirement for Ras in calcium-induced ERK activation has been previously reported (9, 10, 45), we were not able to show functional coupling of Ras to B-Raf, nor was Rap1 activated. Such differences may reflect variability between the properties of PC12 cell clones used (e.g., stably transfected versus wild-type PC12 cells). It is also conceivable that while depolarization may activate Ras, the subsequent actions of Ras may be Raf-independent. Indeed, in a recent report, while Ras was shown to be required for ERK activation by depolarization, no increase in activity of any Raf isoform (A-Raf, B-Raf, c-Raf) was observed (45). One possibility is that the downstream actions of Ras on any of the Raf isoforms may be blocked. A potential inhibitor of Ras is Rap1 itself (46, 47). Rap1 was first identified and perhaps is best characterized as an inhibitor of Ras-dependent signaling (48). Furthermore, Rap1 activation by PKA can account for the ability of cAMP to antagonize Ras signaling (33). Therefore, it is possible that depolarization-induced activation of Rap1 may activate Ras-independent pathways to ERK while inhibiting Ras-dependent pathways.

The signaling pathways stimulated by neuronal calcium are probably determined by a number of factors that regulate cal-

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

Calcium influx. For example, the route of calcium influx may be important (7, 49). Indeed, studies in hippocampal cells have demonstrated that calcium influx via either L-type or N-methyl-d-aspartate channels may have qualitatively different actions on gene expression (50, 51). In the context of ERK activation, the route of calcium entry may also be an important factor in determining signaling events. For example, in an early study, Ras-dependent pathways were identified following ionophore-induced calcium influx (9). In the present study, we show data that suggest different actions of ionophore versus L-type channel calcium influx. Thus, ionomycin stimulated ERKs via a PKA-independent pathway, consistent with a role for Ras, while depolarization activated a PKA-dependent Rap1/B-Raf signaling pathway.

In the present study, we have used three different systems (undifferentiated PC12 cells, neuronal PC12 cells, and hippocampal neurons) to examine depolarization-mediated activation of ERKs. While stimulation of a PKA-dependent pathway to ERKs appears to be a conserved mechanism common to all three cell types, it is possible that differences in calcium flux through L-type channels may differentially control ERK activation. Indeed, in hippocampal cells, depolarization appears to activate equally both PKA-dependent and -independent pathways to ERKs. This may reflect differences in the magnitude of calcium influx, with perhaps large increases in calcium entry via L-type channels leading to activation of both Ras and PKA/Rap1 pathways. Alternatively, the submembrane localization of downstream signaling molecules relative to the source of calcium influx may be an important factor that determines coupling to intracellular signaling cascades (52). For example, in PC12 cells, calcium influx via L-type channels may be very efficiently coupled to PKA-dependent pathways, while in hippocampal cells calcium influx may be able to activate both PKA-dependent and -independent pathways equally. Such a model would be consistent with the emerging theme that compartmentalization of signaling modules can dictate cellular events, particularly with respect to PKA signaling (53, 54).

Ultimately, it is likely that both Ras and Rap1 are important targets for neuronal calcium actions, and the relative contribution of either pathway may be determined by cell type- and stimulus-specific factors. Indeed, the ability of neuronal depolarization to activate both pathways may have important implications in regulating the specificity of calcium-mediated signaling and gene expression, as has been described for NGF (34). Moreover, the ability to activate both Ras- and PKA/Rap1-dependent signaling pathways may allow neurons to translate specific electrical impulses into distinct intracellular signaling events (55). This may be important in regulating the temporal profile of ERK signaling and subsequent transcriptional events (56).

Given our findings that Rap1 may be a target for neuronal calcium, it is important to consider the mechanisms by which it may be activated. In previous studies, we have shown that both cAMP and NGF stimulate Rap1 in a PKA-dependent manner (34, 35). These findings, together with our present data showing that the activation of both Rap1 and its downstream effector, B-Raf, are reversed by inhibition of PKA, argue for a role for PKA-regulated Rap1 activation. Our work with NGF suggests that the Rap1 GEF, C3G, may be important in this context (34). However, recent studies have identified other novel Rap1-GEFs that are activated independently of PKA. Interestingly, these appear to be stimulated directly by both calcium and cAMP binding (32, 37, 38). Moreover, they are highly expressed in localized regions within the CNS. A role for such factors in neuronal calcium-regulated Rap1 activation is therefore entirely possible. Clearly, further studies examining the role of Rap1 activation and, in particular, the relative contribution of specific Rap1-GEFs in neurons are warranted.
A requirement for PKA in mediating the actions of depolarization on Rap1/B-Raf activity suggests a role for calmodulin-stimulated adenylate cyclases (36). Consistent with this, in the present study we have demonstrated a requirement for calmodulin in the actions of depolarization. Calmodulin-stimulated adenylate cyclases are specifically expressed within the CNS and can be activated by depolarization and neuronal activity. In both neurons and PC12 cells, calcium influx has been shown to increase cAMP levels and stimulate PKA activity (36, 57). As such, many activity-dependent neuronal events such as regulation of synaptic plasticity and gene expression appear to require PKA activity (58–64). Moreover, a number of recent reports also describe a role for ERKs in these processes (18–22). Given our present findings, it is possible that some of these actions may reflect the involvement of PKA-dependent Rap1/B-Raf signaling to ERKs. For example, studies examining the mechanisms of long term facilitation in Aplysia have also indicated that regulation of ERK activity by PKA may be an im-

**Fig. 3.** Depolarization of neuronally differentiated PC12 cells and hippocampal neurons activates ERKs via PKA. A, undifferentiated PC12 cells (left panels) and neuronally differentiated PC12 cells (right panels) were treated with KCl (60 mM) for 10 min in the absence or presence of the PKA inhibitor, H89 (10 μM), as indicated. Cells were then lysed, and equal protein amounts per treatment condition were used for Western blot with phospho-ERK1/2 or ERK1/2 antibody. B, immunofluorescence of hippocampal neurons with phospho-ERK1/2 antibody (upper panels) or ERK1/2 antibody (lower panels). Cells were untreated (cont) or treated for 10 min with KCl (60 mM) in the absence (KCl) or presence (KCl + H89) of the PKA inhibitor H89. Shown are representative images of cells treated in parallel. C (i), quantitation of data represented in B (upper panel). Bars indicate intensity of fluorescence in the cell bodies of phospho-ERK1/2-stained neurons expressed as fold increase over untreated cells (mean ± S.E.; n > 25 cells/condition). ii, quantitation of data represented in B (lower panel). Bars indicate intensity of fluorescence in the cell bodies of ERK1/2-stained neurons expressed as fold increase over untreated cells (mean ± S.E.; n > 25 cells/condition). iii, Western blot of hippocampal neurons. Cells were treated as indicated for 10 min in the absence or presence of H89. Cells were then lysed, and equal protein amounts per treatment condition were used for Western blot with phospho-ERK1/2 antibody.

**Fig. 4.** Depolarization activates B-Raf, but not Raf-1, via PKA in PC12 cells. A, PC12 cells were treated with KCl (60 mM, 5 and 20 min) or left untreated (0 min) in the absence or presence of the PKA inhibitor H89 as indicated. B-Raf was immunoprecipitated from equal amounts of lysate per condition, and immune complex assays were performed using MEK-1 as a substrate. A representative autoradiogram with the position of MEK-1 is shown. The bottom panels indicate equal protein amounts of B-Raf per treatment as assayed by Western blot. B, PC12 cells were treated as in A, and Raf-1 immune complex kinase assays were performed following immunoprecipitation of Raf-1. The bottom panels indicate equal protein amounts of Raf-1 per treatment as assayed by Western blot. NGF-treated cells (NGF; 50 ng/ml) served as a positive control. The lower panel shows the phosphorylation of ERKs in the same whole cell lysates, using phospho-specific ERK1/2 antibody.
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In a recent study, it was demonstrated that PKA was specifically required for the nuclear translocation of ERKs in neuronal cells via an as yet undefined mechanism (18). It has been widely established that cytoplasmic stimulation of ERKs is necessary for their subsequent cellular relocalization (65, 66). Hence, our findings suggest that PKA-dependent activation of ERKs themselves may contribute to their nuclear translocation (35). However, based on this, we cannot address nor rule out an additional role for PKA in ERK translocation. Nevertheless, our results together with the earlier described study (18) do point to important multiple roles for PKA in the control of neuronal ERK function.

In conclusion, we have identified a PKA-dependent activation of a Rap1/B-Raf signaling pathway downstream of neuronal depolarization. This pathway may be important in neuronal calcium signaling to ERKs. As such, targeting of a PKA/Rap1/B-Raf pathway may regulate a number of activity-dependent events such as changes in gene expression and synaptic plasticity. Furthermore, since activation of Rap1/B-Raf via PKA is a pathway shared by neurotrophins, cAMP, and depolarization (33–35), we suggest that this pathway may underlie the synergy between these factors in regulating neuronal function.

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