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SHORT COMMUNICATION
Activation of extracellular signal-regulated kinases 1 and 2 by depolarization stimulates tyrosine hydroxylase phosphorylation and dopamine synthesis in rat brain

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Abstract

Production of dopamine is regulated via phosphorylation of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of catecholamines. Here we have used a preparation of rat striatal slices to examine the involvement of two mitogen-activated protein kinases (MAPKs), extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), in the depolarization-dependent regulation of TH phosphorylation and dopamine synthesis. Depolarization with elevated KCl (45 mM) caused an increase in the phosphorylation state and, thereby, activation of ERK1/2. The same stimulus also increased TH phosphorylation at Ser19, Ser31 and Ser40 (measured using site- and phospho-specific antibodies) and TH activity [measured as 3,4-dihydroxyphenylalanine (DOPA) accumulation]. A MAPK/ERK kinase inhibitor, PD098059, decreased the basal levels of phospho-ERK1/2 and prevented the increase in ERK1/2 phosphorylation induced by depolarization. PD098059 also decreased both basal and depolarization-induced phosphorylation of TH at Ser31 and reduced the increase in Ser40 phosphorylation induced by high potassium, but did not affect Ser19 phosphorylation. PD098059 alone inhibited basal TH activity and decreased the accumulation of DOPA induced by depolarization. These data provide evidence for the involvement of ERK1/2 in the regulation of the state of phosphorylation of TH at Ser31 and Ser40 and a correlation between ERK1/2-dependent phosphorylation of TH and stimulation of dopamine synthesis in the brain.

Introduction

Tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, is regulated via phosphorylation at multiple seryl residues. The intracellular pathways and protein kinases involved in the regulation of the state of phosphorylation of each site have been identified in part. Ser19 phosphorylation is stimulated by increases in intracellular calcium concentration (Haycock, 1990); Ser31 phosphorylation is catalysed by two mitogen-activated protein kinases (MAPKs) – extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) – (Haycock *et al*., 1992) and Ser40 phosphorylation is increased via activation of the cAMP pathway (Vulliet *et al*., 1980; Haycock & Haycock, 1991). Previous studies have shown that increases in TH phosphorylation at Ser31 (Haycock *et al*., 1992; Sutherland *et al*., 1993) or Ser40 (Harada *et al*., 1996; Lindgren *et al*., 2000; Lindgren *et al*., 2001) are accompanied by increases in enzymatic activity. In contrast, Ser 19 phosphorylation does not appear to directly regulate TH activity. Although its exact physiological role remains to be elucidated (Sutherland *et al*., 1993; Haycock *et al*., 1998; Lindgren *et al*., 2000), it has been demonstrated recently that Ser19 phosphorylation facilitates the phosphorylation of Ser40 *in vitro* (Bevilaqua *et al*., 2001).

One of the major dopaminergic systems in the brain originates in the substantia nigra pars compacta and projects to the dorsal striatum. Previous studies have shown that electrical stimulation of nigrostriatal fibers increases striatal TH activity (Murrin *et al*., 1976) and dopamine biosynthesis (Murrin & Roth, 1976). These effects are associated (*in vivo*) with concomitant increases in TH phosphorylation at the level of Ser19, Ser31 and Ser40 (Haycock & Haycock, 1991). Stimuli like membrane depolarization (Rosen *et al*., 1994; Impey *et al*., 1998) and electrical stimulation (Sgambato *et al*., 1998) are known to activate ERK1/2 via phosphorylation catalysed by the MAPK/ERK kinase (MEK). In chromaffin cells, ERK1/2 have been shown to phosphorylate Ser31 and increase TH activity (Haycock *et al*., 1992; Halloran & Vulliet, 1994; Bobrovskaya *et al*., 2001) and the MEK/ERK pathway has been implicated in depolarization-dependent activation of TH (Griffiths & Marley, 2001). In fact, ERK-dependent phosphorylation of Ser31 appears to be responsible for the depolarization-dependent increase in TH activity in chromaffin cells (Salvatore *et al*., 2001).

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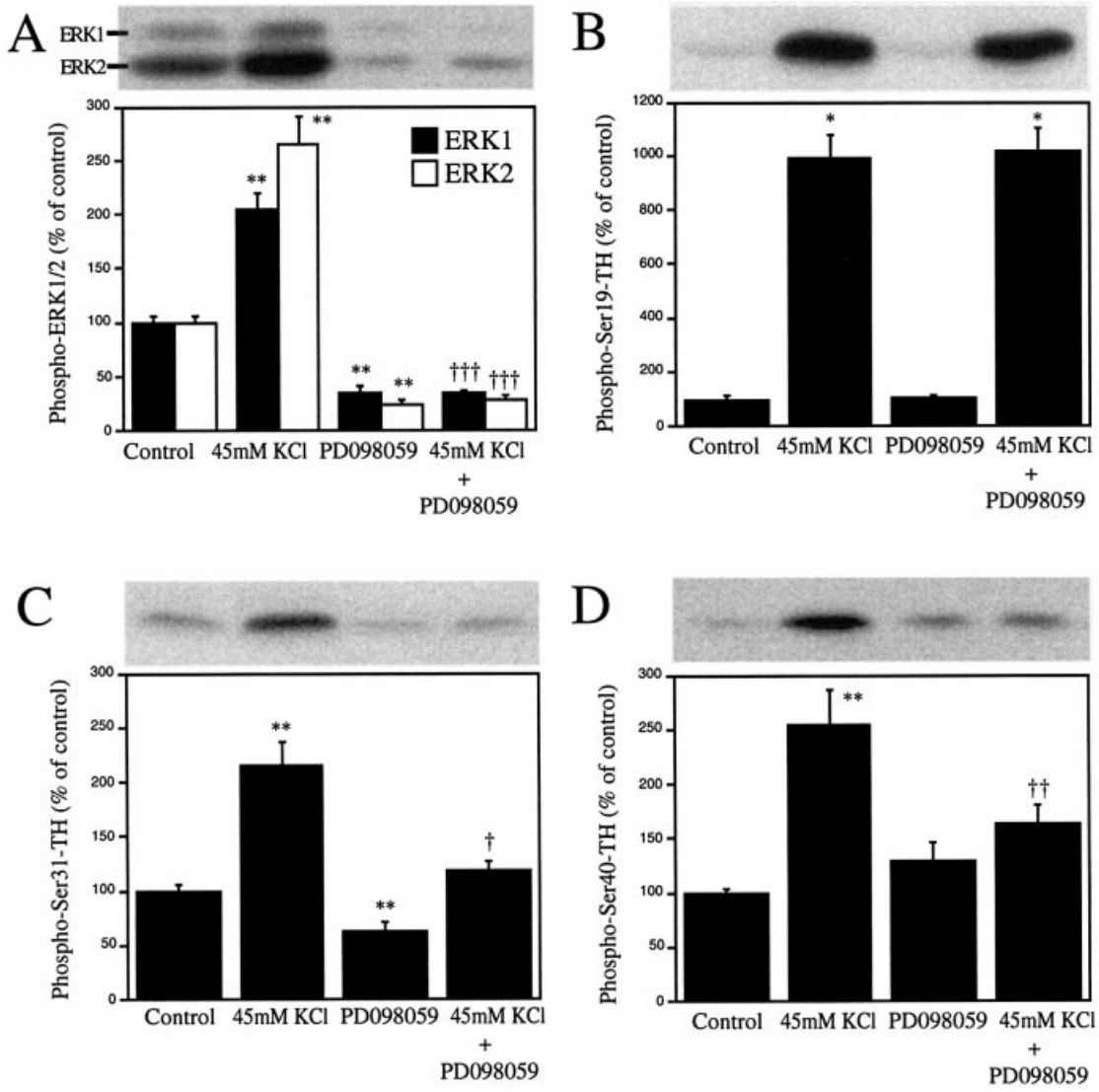

FIG. 1. Effect of 45 mM KCl and PD098059 on the phosphorylation of ERK1/2 (A) and TH (B–D). Striatal slices were preincubated for 15 min in the presence or absence of PD098059 (100 μM) and then incubated for an additional 5 min in the presence of PD098059 plus 45 mM KCl. Upper panels show autoradiograms of typical Western blots obtained using a polyclonal antibody raised against phospho-p44/42 MAP kinase (A), phospho[Ser19]-TH (B), phospho[Ser31]-TH (C) and phospho[Ser40]-TH (D). Lower panels show summaries of four to seven experiments in which immunoreactivities are expressed as percentages of those in the absence of treatment. Bars show mean + SEM values. $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. control, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ interaction between 45 mM KCl and PD098059 treatment, two-way ANOVA.

Very little is known, about the involvement of ERK1/2 in the regulation of TH phosphorylation and dopamine biosynthesis in the brain. In the present study, we examined the involvement of ERK1/2 in the regulation of site-specific phosphorylation and activation of TH produced by high-potassium depolarization.

Materials and Methods

Chemicals

Forskolin, 3,4-dihydroxyphenylalanine (DOPA), PD098059 2[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one and m-hydroxybenzylhydrazine (NSD-1015) were purchased from Sigma (St. Louis, MO, USA). The ECL Plus immunoblotting detection kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The rabbit polyclonal antiphospho-p44/42 MAP kinase (Thr202/Tyr204) was obtained from Cell Signalling Technology (Beverly, MA, USA).

Preparation and incubation of striatal slices

Male, Sprague–Dawley rats (125–175 g; B & K Universal AB, Sweden) were killed by decapitation, and the brains were rapidly removed. For Western blot and DOPA assays, striata were dissected on an ice cold surface, and coronal slices (300 μm) were prepared from each striatum using a McIlwain tissue chopper. Two slices were placed in individual 5-mL polypropylene tubes containing 2 mL of Krebs–Ringer's bicarbonate buffer [KRB (in mM); NaCl, 118; KCl, 4.7; $CaCl_2$, 1.3; $MgSO_4$, 1.5; KH_2PO_4, 1.2; $NaHCO_3$, 25 and glucose, 11.7; equilibrated with 95% O_2/5% CO_2 (v/v), pH 7.3]. The samples were incubated at 30 °C for two 30-min intervals, each followed by replacement of the medium with 2 mL fresh KRB. Test substances were then added for various durations. When PD098059 was used, the slices were preincubated with the drug during the last 15 min of the second 30-min interval. Depolarization was achieved by raising the concentration of KCl in the buffer from 4.7–45 mM. For the determination of DOPA, slices were first incubated for 5 min in the

presence of test substances and then for 15 min in the presence of test substances plus the aromatic L-amino acid decarboxylase inhibitor, NSD-1015 (100 μM). After incubation, the solutions were rapidly removed and the tubes were placed on dry ice until assayed.

Western blot assay of phospho-TH and phospho-ERK

Frozen tissue samples were sonicated in 200 μL of 1% sodium dodecyl sulphate and boiled for 10 min. Aliquots (5 μL) of the homogenate were used for protein content determination using a protein measurement kit (bicinchoninic acid; Pierce Europe; Oud Beijerland, The Netherlands). Equal amounts of protein (containing equal amounts of TH) from each sample were loaded onto 10% polyacrylamide gels, and the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes (Amersham Pharmacia Biotech, Uppsala, Sweden), as described previously (Towbin *et al*., 1979). The membranes were immunoblotted using affinity-purified polyclonal antibodies that selectively detect phospho[Ser19]-TH, phospho[Ser31]-TH, phospho[Ser40]-TH (Salvatore *et al*., 2000) or phospho-p44/42 MAP kinase (Thr202/Tyr204). Antibody binding was revealed by incubation with goat anti-rabbit horseradish peroxidase-linked IgG (diluted 1 : 10 000; Pierce Europe) and the ECL Plus immunoblotting detection method. Chemiluminescence was detected by exposure to X-ray film. Immunoreactivities corresponding to the phospho-TH or phospho-ERK1/2 bands were determined by densitometry, using NIH Image (version 1.61) software. To determine the relative increases in protein phosphorylation, immunoreactivities of the samples were interpolated to standard curves generated by a range of aliquots of pooled tissue standard (striatal slices treated with okadaic acid) run on each gel.

Determination of DOPA

Frozen tissue samples were sonicated in 100 μL of 0.1 mM perchloric acid and centrifuged at 10 000 *g* for 10 min. The pellets were resuspended in 100 μL of sodium dodecyl sulphate and their protein content was determined. The levels of DOPA in the supernatant were determined using HPLC as described previously (Lindgren *et al*., 2001).

Results and discussion

Incubation of striatal slices for 5 min in the presence of 45 mM KCl increased the phosphorylation of ERK1/2 (Fig. 1A). Pre-incubation for 15 min with the MEK inhibitor, PD098059 (100 μM), reduced the basal levels of phospho-ERK1/2 by more than 50% and abolished the depolarization-induced increase in phosphorylation (Fig. 1A). Thus, in agreement with previous studies performed in chromaffin (Rosen *et al*., 1994) and hippocampal (Impey *et al*., 1998) cells, depolarization appears to activate ERK1/2 specifically via MEK-dependent phosphorylation.

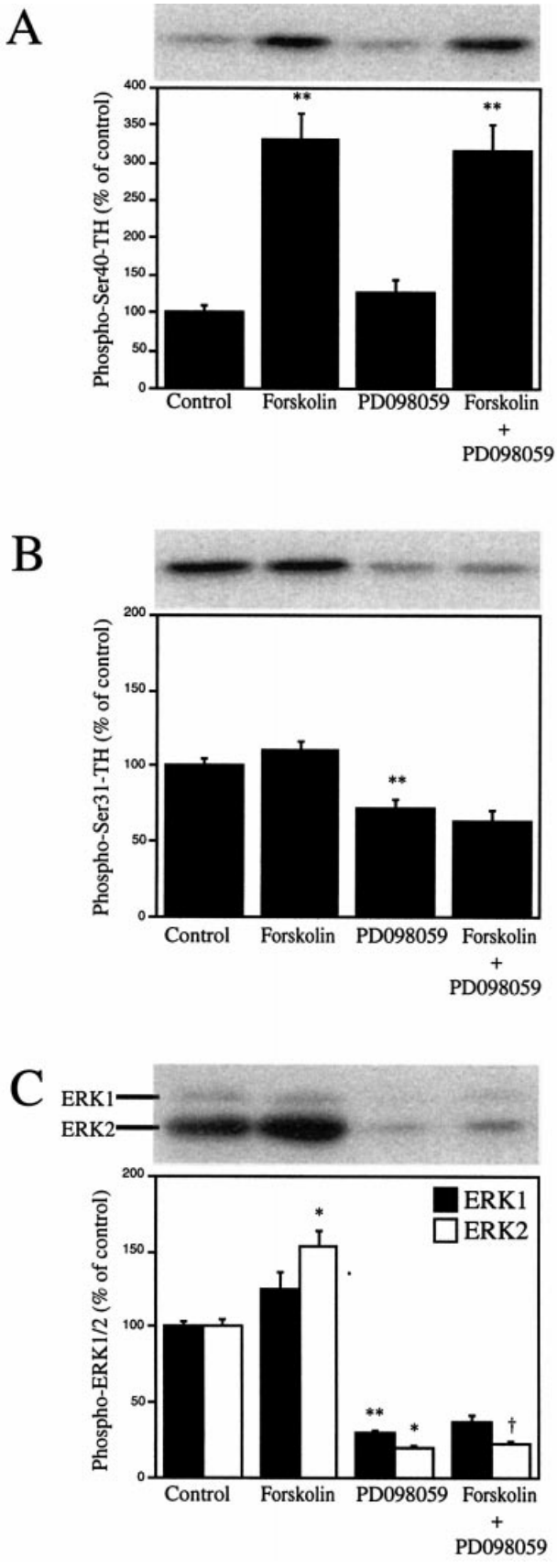

FIG. 2. Effect of forskolin and PD098059 on the phosphorylation of TH (A and B) and ERK1/2 (C). Striatal slices were preincubated for 15 min in the presence or absence of PD098059 (100 μM) and then incubated for an additional 5 min in the presence or absence of PD098059 plus or minus forskolin (1 μM). Upper panels show autoradiograms of typical Western blots obtained using a polyclonal antibody raised against phospho[Ser40]-TH (A), phospho[Ser31]-TH (B) and phospho-p44/42 MAPK (C). Lower panels show summaries of four to seven experiments in which immunoreactivities are expressed as percentages of those in the absence of stimulation. Bars show mean + SEM values. $*P < 0.01$ and $**P < 0.001$ vs. control, $^{\dagger}P < 0.05$, interaction between 45 mM KCl and forskolin treatment, two-way ANOVA.

High potassium also produced large increases in the phosphorylation of TH at Ser19, Ser31 and Ser40 (Fig. 1B–D). The increase in Ser19 phosphorylation was maximal after 2 min of incubation with 45 mM KCl and persisted, unchanged, for at least 20 min (data not shown). The increases in phosphorylation of Ser31 and Ser40 were maximal after 10 min of incubation with 45 mM KCl (287 ± 24% and 285 ± 56% of control, respectively). After 20 min of incubation in the presence of 45 mM KCl, Ser31 was still highly phosphorylated (269 ± 38% of control), whereas Ser40 phosphorylation was significantly reduced (185 ± 49% of control). PD098059 reduced the increases in phosphorylation at Ser31 and Ser40 produced by depolarization (Fig. 1C and D) and significantly decreased the basal levels of Ser31, but not Ser40, phosphorylation (cf. Fig. 1C and D). In contrast, PD098059 did not affect either basal or depolarization-induced phosphorylation of Ser19 (Fig. 1B). Therefore, although the portion of phospho-ERK1/2 immunoreactivity derived from dopaminergic compartments (i.e. nigrostriatal nerve terminals) remains indeterminate, activation of ERK1/2 via MEK appears to play a critical role in the phosphorylation of TH at Ser31 and Ser40, in the striatum.

The involvement of ERK1/2 in the phosphorylation of Ser40 was somewhat unexpected because most evidence indicates that this site is specifically phosphorylated by cAMP-dependent protein kinase (Vulliet *et al*., 1985; Haycock & Haycock, 1991; Lindgren *et al*., 2000; but see also Sutherland *et al*., 1993). In order to exclude the possibility that PD098059 affects Ser40 phosphorylation via inhibition of cAMP-dependent protein kinase, we tested the effect of the MEK inhibitor on the increase in TH phosphorylation induced by forskolin, an activator of the cAMP pathway. As reported previously (Lindgren *et al*., 2000), incubation of striatal slices for 5 min in the presence of 1 μM forskolin produces a large increase in Ser40 phosphorylation (Fig. 2A), without affecting Ser31 phosphorylation (Fig. 2B). PD098059 did not alter the forskolin-mediated increase in Ser40 phosphorylation (Fig. 2A), demonstrating its selectivity for MEK and confirming the specific involvement of ERK1/2 in the stimulation of TH phosphorylation exerted by depolarization at Ser40. Interestingly, forskolin increased the phosphorylation state of ERK2 (Fig. 2C), supporting previous studies which implicate cAMP in the activation of the ERK pathway (Vossler *et al*., 1997). Such an increase, however, was lower than that caused by depolarization (cf. Figs 1A and 2C) and did not produce any changes in Ser31 phosphorylation (Fig. 2B).

The finding that the ERK pathway regulates TH phosphorylation at Ser31 and Ser40, two sites which are critically involved in the control of enzymatic activity (Haycock *et al*., 1992; Sutherland *et al*., 1993; Harada *et al*., 1996; Lindgren *et al*., 2000; Lindgren *et al*., 2001), led to the examination of the involvement of ERK1/2 in the regulation of dopamine biosynthesis. When striatal slices were incubated with PD098059, a significant reduction in the accumulation of DOPA in the presence of NSD-1015 was observed (Table 1). This effect, indicative of a decrease in TH activity, was accompanied by a specific reduction in Ser31 phosphorylation (cf. above; Figs 1C and 2B) [unlike in PC12 cells (Salvatore *et al*., 2001), Ser40 phosphorylation was not affected by PD098059 (Figs 1D and 2A)]. Therefore, ERK1/2-mediated phosphorylation at Ser31 appears to be involved in maintaining basal levels of TH activity.

In agreement with previous studies (Murrin *et al*., 1976; Murrin & Roth, 1976), incubation of striatal slices in the presence of 45 mM KCl produced a large increase in DOPA accumulation (Table 1). PD098059 reduced this effect, indicating the involvement of the ERK pathway in the increase in TH activity produced by depolarization. The MEK inhibitor also decreased the phosphorylation of TH at Ser31 and Ser40 induced by depolarization (Fig. 1C and D). Further studies will be necessary to assess the possible relative contribution of these two sites to the depolarization-induced increase in TH activity.

TABLE 1. Effects of 45 mM KCl and PD098059 on the accumulation of L-DOPA in rat striatal slices

Treatment	Levels of L-DOPA (%)
Control	100 ± 6
45 mM KCl	189 ± 16*
PD098059	73 ± 4*
45 mM KCl + PD098059	115 ± 9†

Slices were incubated for 15 min in the presence of 100 μM PD098059 and for an additional 20 min in the presence of PD098059 plus 45 mM KCl. NSD-1015 (100 μM) was added during the last 15 min of incubation. The samples were then sonicated in 0.1 M perchloric acid and centrifuged. The levels of DOPA recovered in the supernatant were determined by HPLC and normalized according to the protein content (see Materials and Methods; 100% corresponds to 6.7 pmol of DOPA/min per mg protein). The data were obtained from eight experiments performed in duplicate or triplicate and represent mean ± SEM values. *$P < 0.001$ and †$P < 0.05$ interaction between 45 mM KCl and PD098059 treatment, two-way ANOVA.

In conclusion, in striatal slices, activation of ERK1/2 stimulates, whereas inhibition of ERK1/2 reduces, the activity of TH. These effects are accompanied by changes in TH phosphorylation at Ser31 and Ser40, two sites implicated in the regulation of dopamine biosynthesis. Thus, it appears that, in the brain, the MAPK pathway, which has been extensively studied in relation to postsynaptic changes (such as gene expression) (Grewal *et al*., 1999), is also able to regulate short-term presynaptic function.

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Abbreviations

ERK1/2, extracellular signal-regulated protein kinases 1 and 2; DOPA, 3,4-dihydroxyphenylalanine; KRB, Krebs-Ringer bicarbonate buffer; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NSD-1015, m-hydroxybenzylhydrazine; TH, tyrosine hydroxylase.

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