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ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 *in situ*

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ABSTRACT **Tyrosine hydroxylase (TH) is phosphorylated at four sites *in situ* and *in vivo*, and the protein kinases that phosphorylate three of these sites (Ser^8, Ser^{19}, Ser^{40}) have been identified. In intact cells, the phosphorylation of the fourth site (Ser^{31}) is increased in response to phorbol esters or nerve growth factor (NGF). Here, we show that Ser^{31} is phosphorylated by ERK1 and ERK2, two myelin basic protein and microtubule-associated protein kinases. Extracts of NGF- or bradykinin-treated PC12 rat pheochromocytoma cells were fractionated on Mono Q columns. Protein kinase activity toward Ser^{31} in TH was present in two peaks corresponding to myelin basic protein kinase activities previously identified as ERK1 and ERK2. Phosphorylation of purified TH *in vitro* by both kinases was selective for Ser^{31} up to at least 0.6 mol of phosphate per mol of TH subunit. Treatment of intact PC12 cells with bradykinin or NGF increased both the phosphorylation of TH-Ser^{31} *in situ* and the catalytic activity of ERKs (measured subsequently *in vitro* with myelin basic protein as substrate). Pretreatment of the cells with genistein (a protein-tyrosine kinase inhibitor) decreased the bradykinin- but not the NGF-induced changes in both TH-Ser^{31} phosphorylation and ERK activity. Genistein also inhibited the increases in Ser^{31} phosphorylation produced by phorbol dibutyrate, muscarine, and Ba^{2+}. The data indicate that ERK activity is responsible for phosphorylating TH at Ser^{31} in intact cells and suggest that TH-Ser^{31} phosphorylation may be regulated by multiple signaling pathways that converge at or prior to the activation of the ERKs.**

Activation of catecholaminergic tissues increases the activity of tyrosine hydroxylase (TH), the initial and rate-limiting enzyme in catecholamine biosynthesis (1). The increase in TH activity is associated with an increase in its phosphorylation, which is thought to mediate the activation (2). TH is phosphorylated *in situ* at Ser^8, Ser^{19}, Ser^{31}, and Ser^{40} (3), and the phosphorylation of Ser^{19}, Ser^{31}, and Ser^{40} is regulated by neuronal activity in intact mammalian catecholaminergic tissues (4, 5).

In vitro, Ser^{19} and Ser^{40} are substrates for Ca^{2+}/calmodulin-dependent protein kinase II (CaM-PKII), and Ser^{40} is also a substrate for cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) (6–9). *In situ*, agents that promote Ca^{2+} influx (e.g., high extracellular [K^+]) increase Ser^{19} phosphorylation, consistent with mediation by CaM-PKII, and agents that increase cAMP (e.g., forskolin) increase Ser^{40} phosphorylation, consistent with mediation by PKA (3, 4). However, agents that activate PKC [e.g., phorbol 12,13-dibutyrate (PBt_2)] increase Ser^{31} as opposed to Ser^{40} phosphorylation (3), indicating that the involvement of PKC in TH phosphorylation *in situ* is indirect. Thus, an unidentified protein kinase(s) appears to be directly responsible for Ser^{31} phosphorylation.

A physiological role for TH-Ser^{31} kinase activity seems likely in that Ser^{31} phosphorylation is regulated by neuronal activity *in vivo* in dopaminergic nerve terminals in the brain (4) and in perfused adrenal glands (5). Ser^{31} was only recently identified as a phosphorylation site (3), and the amino acid sequences surrounding Ser^{31} (rat, EAVTS^{31}PRF; bovine and human, EAIMS^{31}PRF) offered few clues regarding candidate protein kinases. Unsuccessful candidates include CaM-PKII, PKA, and PKC, and several well-described (8) as well as several recently characterized (4) protein kinases. In the present study, ERK1 and ERK2, members of a family of serine/threonine protein kinases (10) whose activity requires and is regulated by the concomitant phosphorylation of tyrosine and threonine residues on the kinase (10–15), were identified as TH-Ser^{31} kinases *in vitro* and as probable mediators of the phosphorylation of TH at Ser^{31} *in situ*.

MATERIALS AND METHODS

Materials. The peptide KQAEAVTSPR, corresponding to residues 24–33 in rat TH, was synthesized by the Louisiana State University Medical Center Core Laboratories and is designated TH^{24-33}. Purified rat TH from PC12 pheochromocytoma cells (16) was provided by L. Gahn, and PKA by R. Roskoski, Jr. (both at the Louisiana State University Medical Center). ERK1 was purified from nerve growth factor (NGF)-treated PC12 cells (17). ERK2 from epidermal growth factor (EGF)-treated Swiss mouse 3T3 cells was partially purified by Mono Q and Superose 12 chromatography (18–20). Genistein was from GIBCO and ICN. PBt_2 and thapsigargin were from LC Services, Woburn, MA. 2,5-Di-(*tert*-butyl)-1,4-benzohydroquinone (*t*-Bu_2BHQ) was from Fluka.

Cell Cultures. PC12 cells were grown in medium with 3% horse serum and 3% calf serum and were seeded on collagen-coated plates 3–7 days prior to experiments, at which time the cells were ≈70% confluent (19).

Preparation and Chromatography of Cell Extracts. PC12 monolayers in 15-cm dishes were rinsed and preincubated (30 min, 37°C) in 9 ml of incubation solution (P_i deficient Dulbecco's modified Eagle's medium (Sigma D3656)/25 mM $NaHCO_3$/15 mM Hepes, pH 7.4/5 μM $Tris_2P_i$). Then 1 ml of incubation solution containing test substance (750 ng of 2.5S NGF, 1 μM bradykinin, or no addition) was added. Incubation was continued for 5 min, and the cells were rinsed and harvested in ice-cold column buffer (50 mM β-glycerophos-

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Abbreviations: TH, tyrosine hydroxylase; EGF, epidermal growth factor; NGF, nerve growth factor; CaM-PKII, Ca^{2+}/calmodulin-dependent protein kinase II; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; MBP, myelin basic protein; MAP, microtubule-associated protein; *t*-Bu_2BHQ, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone; PBt_2, phorbol 12,13-dibutyrate.

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phate/1.5 mM EGTA/1 mM dithiothreitol/0.1 mM Na_3VO_4, pH 7.3) containing 1 mM benzamidine and 10 μg of leupeptin per ml. The samples were sonicated and centrifuged, and the supernatants were applied to an HR5/5 Mono Q column (Pharmacia) and eluted with a NaCl gradient (19, 20). Soluble extracts (20) of Swiss 3T3 cells were chromatographed on a Mono Q column as above.

Protein Kinase Activity. Aliquots were incubated with substrate in column buffer containing 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (≈2000 cpm/pmol), 10 μM calmidazolium, and 2 μM PKA inhibitor (TTYADFIASGRTGRRNALHD) (19). Reactions (30°C, 10–20 min, 25 μl) were initiated by adding MgATP. ^{32}P incorporation into myelin basic protein (MBP) or TH$^{24-33}$ was measured after spotting on Whatman P81 paper (20). Reactions using purified TH were terminated by adding Laemmli sample buffer and heating. After SDS/PAGE, ^{32}P incorporation was measured by scintillation counting of excised TH bands.

Regulation of TH Phosphorylation and ERK Activity *in Situ*. PC12 cultures (12-well plates) were rinsed and preincubated (60–90 min, 37°C) in incubation solution containing $^{32}P_i$ (0.5–1 mCi/ml; 1 mCi = 37 MBq). Cells were incubated with test substances (or vehicle) and solubilized for SDS/PAGE (3). The ^{32}P-labeled TH band was excised and digested with trypsin, and site-specific phosphorylation was quantitated by in-line radiochemical detection of ^{32}P-labeled phosphopeptides separated by reversed-phase HPLC (3, 5).

In experiments comparing TH-Ser^{31} phosphorylation and ERK activity, cells were seeded in 12-well plates (incubated with $^{32}P_i$) and in 6-well plates (incubated without $^{32}P_i$). After treatment with test substances, the cells were scraped, triturated, and sonicated in ice-cold column buffer containing leupeptin and benzamidine (in lieu of the SDS solution). Ser^{31} phosphorylation was assayed in supernatants from ^{32}P-labeled cells after addition of concentrated Laemmli sample buffer, and MPB kinase activity was assayed in aliquots of the supernatants from unlabeled cells.

Ser^{31} Phosphorylation and TH Activity *in Vitro*. Purified TH (1 μM TH subunit) was incubated at 30°C for 30 min with ≈1 μM catalytic subunit of PKA, ERK1, or ERK2 in column buffer with 10 μM leupeptin and 50 μg of bovine serum albumin per ml, 10 mM $MgCl_2$, and 0.1 mM ATP. [γ-^{32}P]ATP (≈20,000 cpm/pmol) was added for determining the stoichiometry of ^{32}P incorporation. ^{32}P incorporation was determined as above, and the site specificity was determined by reversed-phase HPLC after tryptic digestion of the ^{32}P-TH in gel slices (3).

TH activity (21) and TH protein (52) were determined as described (5). Phosphorylation reactions were quenched by adding 25 vol of cold pH 7.2 assay buffer (50 mM $Tris_2P_i$/30 mM TrisF/2 mM dithiothreitol/0.5 mM EDTA, 2 μM leupeptin with 2000 units of catalase and 50 μg of bovine serum albumin per ml, pH 7.2 at 30°C). The samples were kept at 0–4°C and assayed within 60 min. Replicate 10-μl aliquots were added to 20-μl aliquots of pH 7.2 or pH 6.2 assay buffer containing L-[3,5-^{3}H]tyrosine (1 μCi, 10 μM) and (6*R*)-5,6,7,8-tetrahydro-L-biopterin. Dopamine (5 μM) was included in some reactions. In the pH 6.2 buffer, 50 mM Tris·Mes was substituted for 50 mM $Tris_2P_i$, resulting in a final reaction pH of 6.5 at 30°C. The reaction was initiated by transfer to 30°C and stopped after 15 min by addition of acidified charcoal suspension.

RESULTS

Phosphorylation of TH-Ser^{31} by ERK1 and ERK2 *in Vitro*. Mono Q chromatography of extracts from control and EGF-treated Swiss 3T3 cells gave three EGF-stimulated peaks of TH-phosphorylating activity, which were eluted at 50, 90, and 150 mM NaCl (Fig. 1*A*). The activity in the last two peaks

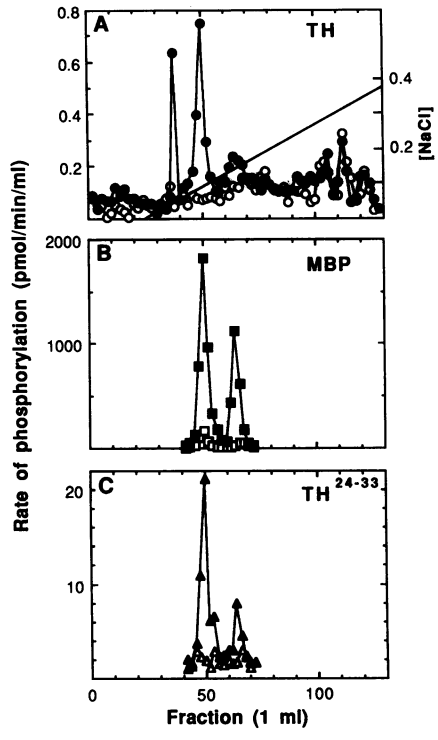

FIG. 1. Coelution of TH and TH$^{24-33}$ kinase activity with Swiss 3T3 cell MBP kinase activity. Soluble extracts of Swiss 3T3 cells treated with EGF (100 ng/ml) (solid symbols) or vehicle (open symbols) were applied to an HR 5/5 Mono Q column and eluted with a NaCl gradient (solid line). Fractions were assayed for kinase activity using purified TH (20 μg/ml) (*A*), MBP (0.33 mg/ml) (*B*), or 1 mM TH$^{24-33}$ (*C*) as substrate.

coincided with two peaks of MBP kinase activity (Fig. 1*B*) that represent ERK2 (90 mM NaCl) and ERK1 (150 mM NaCl), two closely related microtubule-associated protein (MAP)/MBP kinases (14, 15), in Swiss 3T3 cell extracts (18–20). The site specificity of TH phosphorylation by the three peaks revealed a TH-Ser^{31} kinase activity in the second and third peaks but not in the first peak (which produced Ser^{40} phosphorylation). Kinase activity toward TH$^{24-33}$ coincided with the latter two peaks of MBP kinase activity (Fig. 1*C*).

Phosphorylation of TH-Ser^{31} in Intact PC12 Cells. Treatment of PC12 cells with NGF or phorbol ester selectively increases TH-Ser^{31} phosphorylation (3). Additional compounds were tested to maximize Ser^{31} phosphorylation and, by inference, endogenous Ser^{31} kinase activity. Bradykinin, muscarine, and ATP increased ^{32}P incorporation into Ser^{31} (Table 1). Ser^{40} phosphorylation was also increased by ATP, acting presumably via a metabolite at adenosine receptors. This effect of ATP was inhibited by 100 μM theophylline, an adenosine receptor antagonist. Thapsigargin and *t*-Bu_2BHQ (which mobilize $Ca^{2+}{}_i$ from inositol trisphosphate-sensitive pools) but not caffeine (which acts upon different calcium pools) also increased Ser^{31} phosphorylation. NGF and bradykinin were selected for further study.

Identification of TH-Ser^{31} Kinase Activity in PC12 Cells. Mono Q chromatography of extracts from NGF- or bradykinin-treated PC12 cells separated two peaks of MBP kinase activity (Fig. 2 *A* and *B*) as well as two corresponding peaks of TH$^{24-33}$ kinase activity (Fig. 2 *C* and *D*). The peaks of MBP kinase activity from extracts of treated PC12 cells correspond to ERK2 and ERK1, respectively, in order of elution from Mono Q (10, 18). Moreover, the TH$^{24-33}$ and MBP kinase activities in each peak comigrated on Superose 12, and the resulting fractions each phosphorylated Ser^{31} in purified TH (data not shown).

Table 1. Agents that influence Ser^{31} phosphorylation

Treatment	^{32}P incorporation, % control				
	TH	Ser^{8}	Ser^{19}	Ser^{31}	Ser^{40}
NGF (50 ng/ml)	140	—	—	780	—
PBt_2 (300 nM)	130	—	—	620	—
Bradykinin (100 nM)	150	—	—	920	—
Muscarine (100 μM)	—	—	—	330	—
ATP (10 μM)	160	—	—	600	240
Theophylline (100 μM)/ATP	130	—	—	650	—
$BaCl_2$ (2 mM)	145	—	160	350	—
Caffeine (0.1–3 mM)	—	—	—	—	—
Thapsigargin (100 nM)	—	—	—	280	—
t-Bu_2BHQ (10 μM)	—	—	—	250	—

^{32}P-labeled PC12 cells were treated for 5 min, except that theophylline was added 2 min prior to the ATP. ^{32}P incorporation into TH and TH phosphopeptides was normalized to ^{32}P incorporation into total cellular protein (3). Each condition was run in duplicate or triplicate in two to four different experiments. Median values are presented. The distribution of ^{32}P incorporation into the four sites in control cells was Ser^{8}, 22 ± 1; Ser^{19}, 50 ± 3; Ser^{31}, 6 ± 1; Ser^{40}, 22 ± 2 (mean % ± SE, n = 10). —, Within 80–120% of control.

Correlation Between ERK Activity and Ser^{31} Phosphorylation *in Situ*. Phosphorylation of both tyrosine and threonine residues is required for ERK activity (10–15, 19, 22). NGF treatment of PC12 cells increases the phosphotyrosine content of a 44-kDa and a 42-kDa polypeptide band (23, 24), representing ERK1 and ERK2, respectively (10). In the present study, we observed an increase in [^{32}P]phosphotyrosine (25) associated with a 44-kDa and a 42-kDa band in response to either NGF or bradykinin (data not shown). Hence, we tested tyrosine kinase inhibitors [lavendustin A, tyrphostin, methyl 2,5-dihydroxycinnamate, 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid, genistein; GIBCO] for effects on agonist-stimulated phosphorylation of TH-Ser^{31} in PC12 cells. Of these, genistein inhibited Ser^{31} phosphorylation selectively, without influencing Ser^{19} and/or Ser^{40} phosphorylation. Genistein produced a concentration-dependent (10–360 μM; 2- to 15-min preincubation) inhibition (up to 80%) of bradykinin-stimulated Ser^{31} phosphorylation but had little or no effect on TH phosphorylation in samples treated with NGF. To determine whether the changes in TH-Ser^{31} phosphorylation were paralleled by changes in ERK activity, matched unlabeled and ^{32}P-labeled cells were treated with NGF or bradykinin in the presence or absence of genistein. The increase in Ser^{31} phosphorylation produced by either NGF or bradykinin was associated with an increase in ERK activity (Fig. 3). Moreover, the inhibition by genistein of bradykinin-stimulated Ser^{31} phosphorylation was associated with an inhibition of ERK activity. By contrast, genistein had little effect on NGF-stimulated Ser^{31} phosphorylation or on ERK activity (Fig. 3). Smaller effects of NGF (produced by lower concentrations or shorter treatments) were similarly unaffected by genistein.

Genistein also inhibited the increases in Ser^{31} phosphorylation produced by $BaCl_2$, PBt_2, and muscarine (Table 2). Neither the CaM-PKII-mediated (26–28) increases in Ser^{19} phosphorylation produced by Ba^{2+} or bradykinin nor the PKA-mediated increase in Ser^{40} phosphorylation produced by 8-bromo-cAMP was affected by genistein. The small increases in Ser^{40} phosphorylation produced by Ba^{2+} or PBt_2 were inhibited by genistein, but these increases may have occurred secondarily to ERK activation.

Phosphorylation and Activation of TH by ERK1. Purified ERK1 (from NGF-treated PC12 cells) phosphorylated purified TH (from PC12 cells) readily, and the reaction was specific for Ser^{31} (Fig. 4). At 30°C, ^{32}P incorporation was maximal by 30–60 min and stable for >4 hr. Under the standard conditions (30 min, 30°C, 0.1 mM ATP, ≈1 μM ERK1, 1 μM TH subunit), 0.1–0.2 mol of $^{32}P_i$ was incorporated per mol of TH subunit (n = 3). With 0.1 μM TH subunit and 1 mM ATP, a stoichiometry 0.5–0.6 mol of P_i per mol was achieved (n = 2). ^{32}P incorporation was restricted to Ser^{31} at both levels of phosphorylation.

Phosphorylation of either Ser^{31} by ERK1 (≈0.2 mol of P_i per mol of TH subunit) or Ser^{40} by PKA (≈0.3 mol of P_i per mol of TH subunit) under standard conditions increased the catalytic activity of TH (Table 3). However, the increase in TH activity produced by ERK1 was much smaller than that produced by PKA. Phosphorylation by PKA decreased the inhibition of TH activity produced by dopamine, whereas the

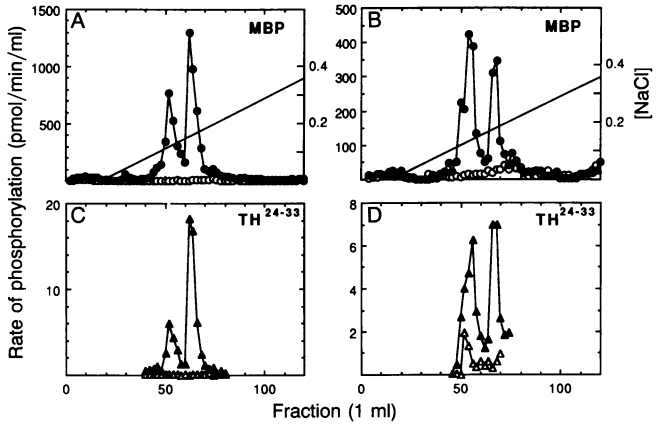

FIG. 2. Coelution of TH^{24-33} kinase activity with PC12 cell MBP kinase activity. Soluble extracts of control (open symbols) or treated (solid symbols) PC12 cells were applied to an HR 5/5 Mono Q column and eluted with a NaCl gradient (solid line). PC12 cells were treated with NGF (75 ng/ml) (*A* and *C*) or 100 nM bradykinin (*B* and *D*). Fractions were assayed with MBP (0.33 mg/ml) or 1 mM TH^{24-33} as substrate.

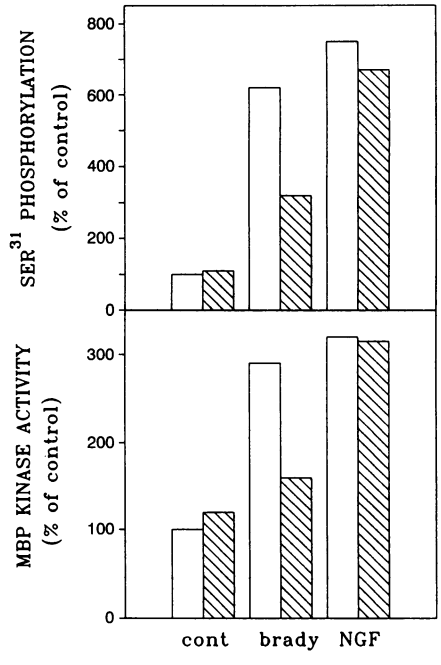

FIG. 3. Coregulation of TH-Ser^{31} phosphorylation and MBP kinase activity in intact PC12 cells. Cultures of PC12 cells were preincubated in the presence (*Upper*) or absence (*Lower*) of $^{32}P_i$. The cells were treated with 200 μM genistein (hatched bars) or vehicle (open bars) for 15 min prior to addition of buffer (control, cont), 100 nM bradykinin (brady), or NGF (50 ng/ml). After 5 min of treatment, the cells were harvested.

Table 2. Inhibition of Ser^{31} phosphorylation by genistein

Test agent	Genistein	^{32}P incorporation, % control		
		Ser^{19}	Ser^{31}	Ser^{40}
NGF (50 ng/ml)	−	—	980	—
	+	—	1080	—
Bradykinin (100 nM)	−	—	890	—
	+	—	260	—
Bradykinin (100 nM, 1 min)	−	240	180	—
	+	215	—	—
Bradykinin (1 μM)	−	140	1210	—
	+	145	320	—
$BaCl_2$ (2 mM)	−	175	350	150
	+	180	—	—
PBt_2 (1 μM)	−	—	920	130
	+	—	130	—
Muscarine (100 μM)	−	—	330	—
	+	—	—	—
8-Br-cAMP (1 mM)	−	—	—	220
	+	—	—	210

^{32}P-labeled PC12 cells were treated with 100 μM genistein (+) or vehicle (−) followed 5 min later by test agent. Incubation was 5 min unless indicated. Ser^8 phosphorylation was within 20% of control in all conditions. Other details are as in the legend to Table 1.

sensitivity of the ERK1-activated enzyme to dopamine was relatively unchanged.

DISCUSSION

Ser^{31} in TH was shown to be a substrate for ERK1 and ERK2 *in vitro*. The activity of the ERKs toward Ser^{31} *in vitro* was produced by treatment of Swiss 3T3 cells with EGF (Fig. 1), treatment of PC12 cells with NGF or bradykinin (Fig. 2), or incubation of inactive ERKs with MAP kinase activator *in vitro* (ref. 19; unpublished work). Moreover, in that changes in ERK activity were associated with changes in Ser^{31}

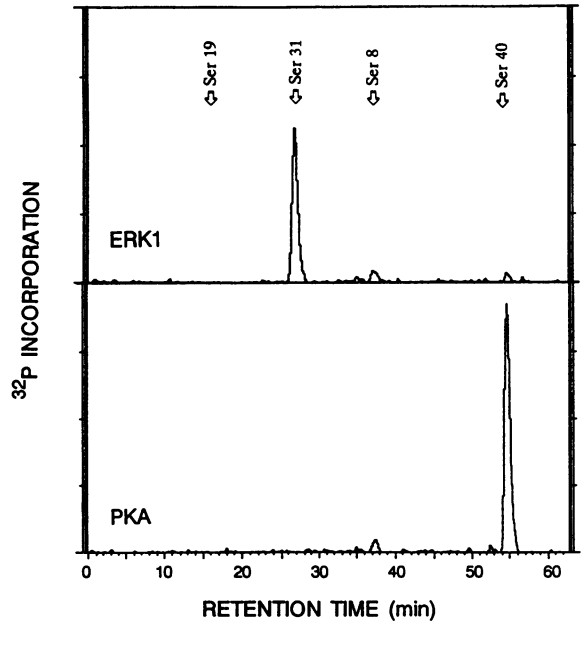

FIG. 4. Site-specific phosphorylation of PC12 TH by ERK1 and PKA. Purified TH was phosphorylated by ERK1 and PKA under the standard conditions. ^{32}P-labeled tryptic TH phosphopeptides were prepared and separated by reversed-phase HPLC on a Vydac C_{18} column (3, 4). The acetonitrile gradient was 0.2%/min. Arrows, retention times of peptides with the indicated phosphorylated residues.

Table 3. Effects of Ser^{31} phosphorylation on TH activity

Assay conditions		TH activity, nmol/(mg·min)		
pH	Addition(s)	Control	ERK1	PKA
6.5	20 μM H_4B	2.7	3.2	13
	200 μM H_4B	7.1	8.6	17
	200 μM H_4B/5 μM DA	1.2	1.6	9.1
7.2	20 μM H_4B	0.7	1.0	11
	200 μM H_4B	3.7	4.7	20
	200 μM H_4B/5 μM DA	0.25	0.33	4.6

Values are the medians of triplicate determinations from one experiment. Comparable results were obtained in three separate experiments. H_4B, tetrahydrobiopterin; DA, dopamine.

phosphorylation, ERKs appear to mediate Ser^{31} phosphorylation *in situ*.

ERK1 and ERK2 belong to a family of serine/threonine protein kinases whose activity requires and is regulated by the concomitant phosphorylation of tyrosine and threonine residues in the kinase (10, 13). ERK activity toward *in vitro* substrates such as MAP, MBP, and S6 kinase II is increased by treatment of diverse cells with a variety of extracellular messengers, including NGF (11, 14, 15). When the NGF- and phorbol ester-sensitive phosphorylation site in TH was initially identified as Ser^{31} (-EAVTS^{31}PRF-) (3), a requirement for proline on the carboxyl side of a serine/threonine phosphorylation site had been described for two protein kinases (29), yeast $p34^{cdc2/CDC28}$ and a PC12 enzyme recently identified as a mammalian *cdc2* gene product (30). [The latter enzyme phosphorylates TH *in vitro*, but at Ser^8 as opposed to Ser^{31} (30).] Phosphorylation of MBP by MAP kinase (ERK2) was shown to occur at Thr^{97} (31), which is immediately N-terminal to a proline residue, and further studies have suggested that a proline two positions N-terminal to the site [i.e., *P*X(S/T)P] is also important for substrate recognition (32, 33). The present data show, however, that the upstream proline is not required absolutely.

Numerous receptor systems in PC12 cells can stimulate ERK activity/Ser^{31} phosphorylation (Table 1 and ref. 3). Muscarinic (but not nicotinic), purinergic P_2 (but not P_2 or adenosine A_1 or A_2), NGF, and bradykinin receptor activation all increase Ser^{31} phosphorylation. However, only two intracellular signaling pathways are needed to accommodate the observed ERK activation/Ser^{31} phosphorylation despite the diversity of effective ligands—an NGF-stimulated pathway and a pathway leading from PKC activation for the rest of the compounds. For example, bradykinin, muscarine, and ATP each have G protein-linked receptors, and each has been shown to increase phosphatidylinositol phosphate turnover and activate PKC in chromaffin and/or PC12 cells. Ba^{2+}, acting intracellularly, increases inositol phosphate turnover and activates PKC, and its effects on Ser^{31} phosphorylation are potentiated by LiCl (J.W.H., unpublished work). Thapsigargin and benzohydroquinone, acting more proximal to PKC, mobilize Ca^{2+} directly from inositol phosphate-sensitive pools (34, 35), and PBt_2 directly activates PKC. Thus, PKC activation may be a common transduction event upon which all of the signals except NGF converge in activating the ERKs. Similar distinctions between phorbol ester-sensitive and -insensitive activation of ERK (36, 37) and between bradykinin and growth factor pathways (38, 39) have been made previously. In support of this hypothesis, down-regulation of PKC by pretreatment with PBt_2 inhibits the bradykinin- and muscarine-induced effects on Ser^{31} phosphorylation (J.W.H., unpublished work). Also, although typically described as a selective inhibitor of protein-tyrosine kinases, genistein inhibits PKC in the range of concentrations used (40). This could account for the inhibition by genistein

of Ser^{31} phosphorylation produced by bradykinin, muscarine, Ba^{2+}, and PBt_2 (but not that by NGF).

The early sequelae responsible for the NGF-stimulated increases in Ser^{31} phosphorylation are less clear. The high-affinity NGF receptor has been identified as the *trkB* protooncogene product, and NGF treatment of PC12 cells increases its tyrosine phosphorylation (41–45). Although this establishes a distinction from the G protein-linked receptors for the other extracellular signals, subsequent biochemical sequelae in the PC12 cells include tyrosine phosphorylation of phospholipase C-γ, increased diacylglycerol levels, and activation of PKC (44–47). As such, these data support a potential involvement of PKC activity in NGF's effects on Ser^{31} phosphorylation. However, down-regulation of PKC fails to block either the Ser^{31} phosphorylation (48) or the MAP kinase activation (36) produced by NGF. Such data would not be contradictory if NGF were to activate a pool of PKC that was compartmentally isolated from the cascade, in which case the convergence of the signaling pathways leading to ERK activation/Ser^{31} phosphorylation could occur downstream of PKC activation. One potential locus is the MAP kinase activator (18), which is activated by bradykinin and NGF (19); however, convergence at the kinases themselves could occur as well.

TH activity is increased directly by Ser^{40} phosphorylation *in vitro* whereas Ser^{19} phosphorylation is thought to be permissive to activation by 14-3-3 (previously referred to as "activator protein") (see ref. 5 and references therein). The phosphorylation of Ser^{31} *in vitro* has not been demonstrated previously, but treatment of PC12 cells with either bradykinin or NGF has been shown to increase TH activity measured subsequently *in vitro* (49–51). In one study (51), a 40–50% increase in TH activity was associated with an increase specifically in Ser^{31} phosphorylation. In the present study, TH activity was increased 20–40% after Ser^{31} phosphorylation; however, it is difficult to assign a physiological significance to this effect in light of the 2- to 15-fold increases in TH activity produced by PKA. Alternatively, the functional consequences of ERK activation/TH phosphorylation may result indirectly by modulating effects produced by another signaling pathway whose activity also reflects increased levels of secretory activity (hence a need for higher rates of tyrosine hydroxylation). For example, Ser^{31} phosphorylation could affect TH activity by altering the effects of cAMP/PKA/Ser^{40} phosphorylation or Ca^{2+}/CaM-PKII/Ser^{19} phosphorylation.

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