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Depolarization-stimulated catecholamine biosynthesis: involvement of protein kinases and tyrosine hydroxylase phosphorylation sites *in situ*

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Abstract

Depolarizing stimuli increase catecholamine (CA) biosynthesis, tyrosine hydroxylase (TH) activity, and TH phosphorylation at Ser19, Ser31, and Ser40 in a Ca^{2+}-dependent manner. However, the identities of the protein kinases that phosphorylate TH under depolarizing conditions are not known. Furthermore, although increases in Ser31 or Ser40 phosphorylation increase TH activity *in vitro*, the relative influence of phosphorylation at these sites on CA biosynthesis under depolarizing conditions is not known. We investigated the participation of extracellular signal-regulated protein kinase (ERK) and cAMP-dependent protein kinase (PKA) in elevated K^+-stimulated TH phosphorylation in PC12 cells using an ERK pathway inhibitor, PD98059, and PKA-deficient PC12 cells (A126-B1). In the same paradigm, we measured CA biosynthesis. TH phosphorylation stoichiometry (PS) was determined by quantitative blot-immunolabeling using site- and phosphorylation state-specific antibodies. Treatment with elevated K^+ (+58 mM) for 5 min increased TH PS at each site in a Ca^{2+}-dependent manner. Pretreatment with PD98059 prevented elevated K^+-stimulated increases in ERK phosphorylation and Ser31 PS. In A126-B1 cells, Ser40 PS was not significantly increased by forskolin, and elevated K^+-stimulated Ser40 PS was three- to five-fold less than that in PC12 cells. In both cell lines, CA biosynthesis was increased 1.5-fold after treatment with elevated K^+ and was prevented by pretreatment with PD98059. These results suggest that ERK phosphorylates TH at Ser31 and that PKA phosphorylates TH at Ser40 under depolarizing conditions. They also suggest that the increases in CA biosynthesis under depolarizing conditions are associated with the ERK-mediated increases in Ser31 PS.

Keywords: blot immunolabeling, Ca^{2+}/calmodulin-dependent protein kinase II, cAMP-dependent protein kinase, extracellular signal-regulated protein kinase, PC12, PD98059.

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Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine (CA) biosynthesis. Treatments that mimic depolarization *in situ* increase CA biosynthesis, TH activity, and phosphorylation of TH in a Ca^{2+}-dependent manner (Yanagihara *et al.* 1984; Waymire *et al.* 1988; Mitchell *et al.* 1990; Haycock and Wakade 1992). Electrical stimulation of the medial forebrain bundle also increases CA biosynthesis, TH activity, and phosphorylation at Ser19, Ser31, and Ser40 in the striatum (Haycock and Haycock 1991). Prolonged electrical stimulation does not deplete cellular CA levels except in the presence of a TH inhibitor (Wakade 1988; Wakade *et al.* 1988). Thus, depolarizing stimulation appears to activate one or more Ca^{2+}-influx-activated protein kinases that phosphorylate TH at specific sites. The phosphorylation of one or more of these sites increases TH activity and CA biosynthesis.

The identity of the protein kinases that phosphorylate TH under depolarizing conditions and the site or sites whose

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Abbreviations used: CA, catecholamine; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CAMKIV, Ca^{2+}/calmodulin-dependent protein kinase IV; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated protein kinase 1 and 2; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; PKA, cAMP-dependent protein kinase; PS, phosphorylation stoichiometry; SDS, sodium dodecyl sulfate; TH, tyrosine hydroxylase; tTH, total tyrosine hydroxylase.

phosphorylation regulates TH activity and CA biosynthesis are not known. Extracellular signal-regulated protein kinase (ERK) can be activated by depolarizing stimuli in a Ca^{2+}-dependent manner (Pavlovic-Surjancev *et al.* 1992; Frodin *et al.* 1995; Rosenblum *et al.* 2000) and is the only protein kinase known to phosphorylate Ser31 both *in vitro* and *in situ* (Haycock *et al.* 1992; Sutherland *et al.* 1993; Thomas *et al.* 1997). Treatments that increase Ser31 phosphorylation alone, such as nerve growth factor (NGF) treatment (Haycock 1990), increase TH activity (Mitchell *et al.* 1990). Ser31 phosphorylation may also increase CA biosynthesis under depolarizing conditions. In cells transfected with TH mutated at Ser40, treatment with elevated K^+ increases CA biosynthesis in a manner comparable to that observed in cells transfected with wild-type TH (Harada *et al.* 1996). Increases in Ser19 phosphorylation do not appear to directly influence TH activity or CA biosynthesis *in situ* (Haycock *et al.* 1998; Lindgren *et al.* 2000). Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) phosphorylates both Ser19 and Ser40 in vitro (Campbell *et al.* 1986; Funakoshi *et al.* 1991; Sutherland *et al.* 1993; Alterio *et al.* 1998) and is also activated by depolarizing stimulation (Colbran 1992; Hanson and Schulman 1992). Although the site on TH phosphorylated by CAMKII *in situ* is unknown, it has been long proposed that CAMKII phosphorylation of TH is involved in depolarization-stimulated CA biosynthesis. A CAMKII inhibitor, KN-93, has been shown to inhibit depolarization-stimulated TH phosphorylation and CA biosynthesis (Sumi *et al.* 1991). cAMP-dependent protein kinase (PKA), shown to phosphorylate Ser40 exclusively *in vitro* (Vulliet *et al.* 1980; Funakoshi *et al.* 1991; Sutherland *et al.* 1993), can also have its activity increased under depolarizing conditions (Shimizu *et al.* 1970; Baizer and Weiner 1985; Keogh and Marley 1991). Increases in Ser40 phosphorylation are associated with increases in TH activity and CA biosynthesis not only *in situ* [in chromaffin and PC12 cells (Haycock 1990; Waymire *et al.* 1991)] and striatal slices (Lindgren *et al.* 2000), but *in vivo* as well (Lew *et al.* 1998). However, in addition to being phosphorylated by CAMKII and PKA, Ser40 is also phosphorylated *in vitro* by protein kinase C (Albert *et al.* 1984; Funakoshi *et al.* 1991), protein kinase G (Roskoski *et al.* 1987), and mitogen-activated protein kinase-activated kinases 1 and 2 (Sutherland *et al.* 1993). Of these protein kinases, only PKA has been shown to directly phosphorylate Ser40 *in situ* (Haycock 1996b). These studies indicate that the cellular mechanism by which depolarization increases CA biosynthesis is by either (i) CAMKII-mediated phosphorylation of Ser40, (ii) ERK-mediated phosphorylation of Ser31, (iii) PKA-mediated phosphorylation of Ser40, or (iv) Ser40 phosphorylation by a protein kinase other than CAMKII or PKA.

The phosphorylation stoichiometry (PS) of TH *in vivo* has been reported recently (Salvatore *et al.* 2000), and site-specific TH PS in PC12 cells (Haycock *et al.* 1998) is comparable to that observed in the nigrostriatal and mesolimbic systems (Salvatore *et al.* 2000). Thus PC12 cells are an appropriate model in which to study the regulation of TH phosphorylation and its relationship to CA biosynthesis. We used two PC12 cell lines, one deficient in the capacity to stimulate PKA activity (van Buskirk *et al.* 1985; Ginty *et al.* 1991). The major objectives were to determine the involvement of CAMKII, ERK, and/or PKA in depolarization-stimulated site-specific TH phosphorylation and in CA biosynthesis.

Materials and methods

Cell culture

PC12 and A126-B1 cells (van Buskirk *et al.* 1985) were propagated on 75-cm^2 tissue culture flasks (Falcon, Franklin Lakes, NJ, USA) pretreated with 2 $\mu g/cm^2$ human placental collagen (Sigma, St Louis, USA; catalog no. C 7521). The growth medium was a 1 : 1 combination of Dulbecco's modified Eagle medium/F12 (Gibco BRL, Gaithersburg, MD, USA; catalog no.10565–018) and OPTI-MEM I reduced serum medium (Gibco BRL; catalog no. 51985–034) containing 5% heat-inactivated fetal bovine serum (Gibco BRL), 5% heat-inactivated donor horse serum (JRH Biosciences, Lenexa, KS, USA), and penicillin (25 U/mL)/streptomycin (25 μg/mL) (Gibco BRL). For experiments, cells were subcultured onto either 12- or 24-well tissue culture plates [Falcon or Costar (Cambridge, MA, USA)], pretreated with 2 $\mu g/cm^2$ human placental collagen, in the same media composition used for propagation. Incubation conditions were 36–37°C in a humidified atmosphere of 6.0–6.5% CO_2.

Drugs and reagents

Forskolin was purchased from Tocris (Baldwin, MO, USA). NGF (rat recombinant) was purchased from Sigma. KN-92 and KN-93 were purchased from Calbiochem (La Jolla, CA, USA) and Seikagaku Corporation (Tokyo, Japan). PD98059 was purchased from Calbiochem. Dimethyl sulfoxide (DMSO) (cell culture reagent grade) was purchased from Sigma (St Louis, MO, USA). All drugs, with the exception of NGF, were dissolved into DMSO to stock concentration and diluted accordingly into treatment buffers.

General experimental conditions

Upon reaching at least 50% confluence, cells were washed with phosphate-buffered saline (Gibco BRL) and pre-incubated (60–90 min) in pH 7.3–7.4 buffer (124 mM NaCl, 25 mM $NaHCO_3$, 11 mM glucose, 10 mM HEPES, 4 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, and 1.2 mM KH_2PO_4) used throughout the experimental procedures. After pre-incubation, the buffer was aspirated and replaced for 10–30 min of pretreatment with selected compounds. In all experiments, theophylline (100 μM; Sigma) was added to each well for the final 10 min of pretreatment to reduce adenosinergic stimulation of cellular cAMP levels (Roskoski and Roskoski 1989). Treatments were initiated by adding buffer or K^+ buffer (in which 58 mM NaCl was replaced with 58 mM KCl) containing, as indicated in the Results section, additional compounds and/or L-[1–^{14}C]tyrosine, as appropriate, for up to 10 min (see Results). During treatment, buffer temperature was maintained at 33–36°C.

Two methods were used to stop treatment effects. In experiments wherein only TH PS was determined, reactions were stopped by aspirating treatment buffer and adding 1% sodium dodecyl sulfate (SDS) solution (pH 8.3 with ~5 mM Tris-HCl and 1 mM EDTA). Solubilized total cellular protein was harvested with trituration and heated for ~5 min. In experiments wherein both CA biosynthesis and TH PS were determined, reactions were stopped by adding an equal volume of 0.4 mM perchloric acid. Cells were triturated and wells were scraped to suspend attached protein. The entire sample was harvested, and precipitated protein was recovered by centrifugation, solubilized in 1% SDS/Tris/EDTA, neutralized with pH 8.9 Tris-HCl, and heated for 5 min. Total protein in each sample, stopped by either method, was determined using the bicinchoninic acid method (Smith *et al.* 1985).

Primary antibodies and blot immunolabeling standards

The characterization of the primary antibodies and standards used to assay tTH and TH PS have been previously described (Haycock *et al.* 1998; Salvatore *et al.* 2000). Primary antibodies used to detect CAMKII were a goat polyclonal antibody generated against the N-terminal region of CAMKII (Calbiochem, San Diego, CA, USA) and a mouse monoclonal antibody (Transduction Laboratories, Lexington, KY, USA). Both were diluted to 1 μg/mL for assay. Ca^{2+}/calmodulin-dependent protein kinase IV (CAMKIV) levels were analyzed using a mouse monoclonal antibody and Jurkat cell lysate (as a positive control) from Transduction Laboratories. Rabbit antibody specific for ERK1/ERK2 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and used to determine total ERK1/ERK2. Rabbit antibody used for detection of diphosphorylated ERK (Haycock 1996a) was purchased from Promega (Madison, WI, USA).

Blot immunolabeling and determination of tTH and TH PS

As described in detail (Haycock 1993b; Salvatore *et al.* 2000), aliquots of samples (normalized for total protein) were subjected to SDS–polyacrylamide gel electrophoresis. For each assay of tTH or TH PS, a range of calibrated standards was included in the electrophoresis run. After electrophoresis, sample proteins were transferred overnight in Tris/glycine/methanol buffer onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Uniformity of protein transfer was verified by Ponceau S staining of the nitrocellulose membranes. The membranes were immersed in quenching buffer containing 1% polyvinylpyrrolidone and 0.05% Tween 20 for a minimum of 2 h before incubation with primary antibody for 1–2 h, 1 h incubation with secondary antibody (as appropriate: swine anti-rabbit IgG and rabbit anti-mouse IgG_1, 0.5 μg/mL, Dako, Glostrup, Denmark; rabbit anti-goat IgG, 0.8 μg/mL, Accurate Chemical and Scientific Corp., Westbury, NY, USA), and 1 h incubation with [^{125}I]protein A (specific activity ~30 μCi/μg; Amersham, Piscataway, NJ, USA).

tTH and site-specific phospho-TH values were determined in separate blots for each primary antibody by quantifying gamma radioactivity per sample and interpolating against the standard curve generated for each assay, as described (Salvatore *et al.* 2000). TH PS for each site was obtained by dividing ng of phospho-TH by ng of tTH.

CA biosynthesis assay

All CA biosynthesis experiments were done in 24-well cell culture plates (CoStar). The method used to quantify evolved [^{14}C]CO_2 after termination of treatments was adapted from previous procedures (Meligeni *et al.* 1982; Cheah *et al.* 1999). L-[1–^{14}C]Tyrosine (specific activity 54 μCi/μmol; Moravek Biochemicals Inc., Brea, CA, USA) was dried under nitrogen and reconstituted in treatment buffers to generate 0.5 μCi/mL. After 30 min pretreatment, each well was fitted with a section of Tygon tubing to collect [^{14}C]CO_2 evolved during treatment (as illustrated in Cheah *et al.* 1999). An equal volume of buffer or K^+ buffer containing L-[1–^{14}C]tyrosine, with or without treatment compound, was added to initiate treatment. After perchloric acid was added to terminate treatment, a rubber stopper fitted with a suspended plastic well containing Whatman-3 filter paper, saturated with Soluene-350 (Packard, Meriden, CT, USA), was placed immediately into the tubing. After allowing absorption of the evolved CO_2 for 45 min, the filter paper was transferred into scintillation cocktail (Hionic-Fluor, Packard) and [^{14}C]CO_2 was determined. Background was determined by collecting [^{14}C]CO_2 evolved under the same experimental conditions, but in the absence of plated cells. For 5 min of treatment, the ratio of experimental sample cpm to background cpm ranged consistently between two- and 10-fold. The background-subtracted [^{14}C]CO_2 value for each sample was normalized to tTH in the inherently paired perchloric acid pellet. Such [^{14}C]CO_2 data have been shown to reflect CA biosynthesis rates (Meligeni *et al.* 1982), and the levels of tTH and THPS recovered from perchloric acid pellets are equal to those from matched samples prepared by the direct SDS-solubilization procedure described above (J. W. Haycock, unpublished observations).

Statistical analyses

In experiments where only blot immunolabeling was analyzed, a paired Student's *t*-test ($p < 0.05$) was used when only two treatment groups were studied, and a repeated measures ANOVA and subsequent least significant difference tests were used when more than two treatment groups were studied ($p < 0.05$).

In experiments wherein TH PS and CA biosynthesis were both analyzed, treatment-associated TH PS and evolved-CO_2 values were divided by the operationally matched control values to determine treatment effects on TH PS and CA biosynthesis (expressed as percent of control). Both dependent measures were taken from the same well of cells. All data presented reflect observations from at least two independent experiments. A repeated measures ANOVA test was used to determine significant differences in the effects of elevated K^+ treatment and PD98059 pretreatment for each cell line. Thus, a total of four groups (buffer, with and without PD98059 pretreatment; K^+ buffer, with and without PD98059 pretreatment) were analyzed for each cell line. If a significant difference ($p < 0.05$) was detected among these four groups, a Bonferroni's multiple comparison test was applied to identify significant differences between group means ($p < 0.05$). Tukey's multiple comparison test and Student's paired *t*-test were used to determine significant differences ($p < 0.05$) in the results from the forskolin and NGF experiments.

Results

TH phosphorylation stoichiometry in PC12 and A126–B1 cells

Basal TH PS at each phosphorylation site in both cell lines is

Table 1 Basal TH PS and total TH in PC12 and A126-B1 cells

	TH PS (mol phosphate/mol site)	
TH phosphorylation site	PC12	A126-B1
Ser19	0.049 ± 0.004	0.041 ± 0.004
Ser31	0.088 ± 0.008	0.073 ± 0.007
Ser40	0.033 ± 0.003	0.010 ± 0.001*
Total TH (ng/μg protein)	18.7 ± 0.8	14.2 ± 0.4*

Both cell lines were subcultured on the same day and harvested 4 days later, after pre-incubation and pretreatment. Samples were processed and quantitative blot immunolabeling was performed as described under Materials and methods. Each of the TH PS ($n = 5$) and total TH ($n = 48$) values is presented as mean ± SEM. *$p < 0.05$ versus PC12 value.

presented in Table 1. There were no significant differences in TH PS at Ser19 or Ser31 between the two cell lines. However, Ser40 PS was three- to four-fold lower in A126-B1 cells than in PC12 cells. The lower Ser40 PS in the A126-B1 cell line is consistent with the diminished PKA activity described in this cell line (van Buskirk *et al.* 1985). Total TH in each cell line is also presented in Table 1. A126-B1 cells had about 25% less tTH per protein than did the PC12 cells.

Effects of elevated K^+ on TH PS in PC12 and A126-B1 cells

In both cell lines, elevated K^+ treatment increased Ser19 PS and Ser31 PS two- to four-fold (Fig. 1) and Ser40 PS about 1.4-fold (Fig. 2a). Despite the same fold increase in Ser40 PS between the two cell lines, the absolute increase in Ser40 PS after treatment with elevated K^+ differed by about five- to seven-fold between the two cell lines (in PC12, 0.020; in A126-B1, 0.003). The absolute values of Ser40 PS in elevated K^+-treated cells were 0.056 in PC12 cells and 0.011 in A126-B1 cells (Fig. 2a). Elevated K^+-stimulated increases in TH PS required extracellular Ca^{2+}: When $CaCl_2$ was omitted from the control and elevated K^+ buffers, there was no increase in PS at any phosphorylation site after 1, 3, or 5 min of elevated K^+ treatment (Table 2).

The extent to which PKA might be involved in the K^+-dependent increase in Ser40 PS in A126-B1 cells was tested by treating the cells with forskolin, a robust activator of adenylyl cyclase. In PC12 cells, relatively low concentrations of forskolin increased Ser40 PS (Fig. 2b), up to three-fold at 0.3 μM (data not shown). In contrast, Ser40 PS in

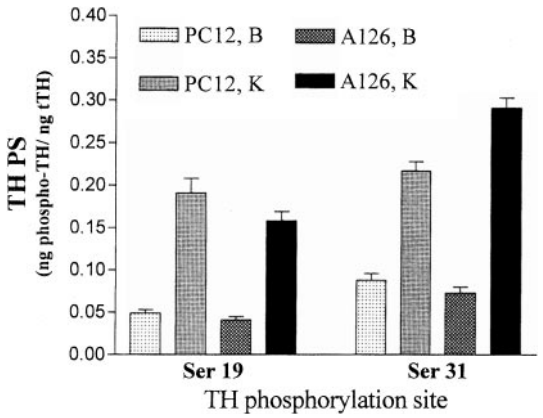

Fig. 1 Effects of elevated K^+ on Ser19 PS and Ser31 PS. After the pretreatment period, PC12 and A126-B1 (A126) cells were treated for 5 min with buffer (B) or elevated K^+ buffer (K), as indicated in the legend. The samples were processed for quantitative blot immunolabeling as described under Materials and methods. Each bar and associated error bar represents the mean ± SEM from one experiment ($n = 5$). Comparable results were obtained in a total of four independent experiments.

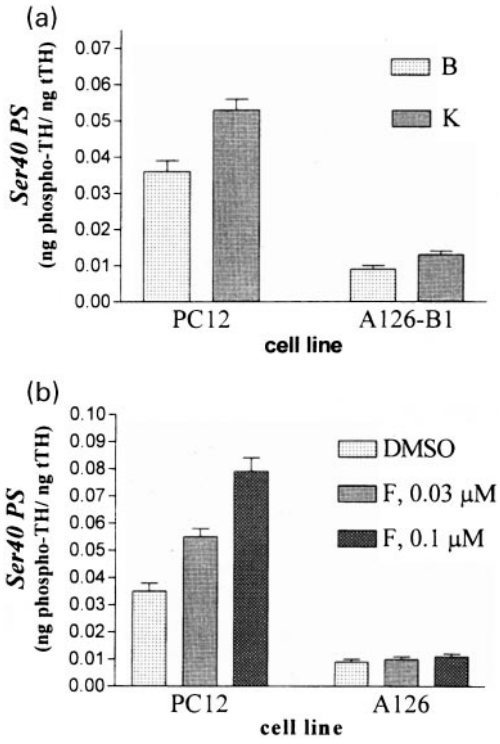

Fig. 2 Effects of elevated K^+ and forskolin on Ser40PS in PC12 and A126-B1 cells. Each bar and associated error bar represents the mean ± SEM. (a) After pretreatment, PC12 and A126-B1 (A126) cells were treated for 5 min with buffer (B) or elevated K^+ buffer (K), as indicated in the legend ($n = 22$ from five separate experiments). (b) After pretreatment, PC12 and A126-B1 (A126) cells were treated for 5 min with buffer (DMSO, as vehicle control) or forskolin-containing buffer (F), as indicated in the legend ($n = 29$ from 5 separate experiments). The samples were processed for quantitative blot immunolabeling as described under Materials and methods. Non-stimulation of Ser40 PS from forskolin treatment of A126-B1 cells was verified in four of the five experiments.

Table 2 Effects of extracellular Ca^{2+} on elevated K^+-stimulated TH PS in PC12 cells

	TH PS (% of basal TH PS)		
Site	*K* ± (min)	0 mM $CaCl_2$	2 mM $CaCl_2$
Ser19	1	93 ± 3	311 ± 27
	3	113 ± 8	273 ± 18
	5	103 ± 9	253 ± 10
Ser 31	1	88 ± 9	102 ± 4
	3	102 ± 4	124 ± 16
	5	107 ± 4	161 ± 15
Ser40	1	90 ± 10	140 ± 8
	3	113 ± 14	149 ± 6
	5	109 ± 8	157 ± 7

PC12 cells were processed as in Table 1 with the exception that after pretreatment, elevated K^+ buffer either without (0 mM $CaCl_2$) or with (2 mM $CaCl_2$) was added for the indicated durations. Each of the values is presented as mean ± SEM ($n = 4$) percentage of basal TH PS determined as in Table 1. Omission of $CaCl_2$ from the buffer during the last 10 min of pretreatment caused a slight, but significant, reduction in basal Ser19 PS.

A126-B1 cells was unaffected by forskolin (Fig. 2b), up to 0.3 μM (data not shown), thus demonstrating the inability of PKA to be activated in these cells and suggesting that the elevated K^+-stimulated increase in Ser40 PS in PC12 cells is mediated, at least in large part, by PKA. The comparatively small increase in Ser40 PS (0.003) in A126-B1 cells from elevated K^+ treatment could be related to phosphorylation by other protein kinases shown to phosphorylate Ser40 *in vitro,* such as CAMKII.

Ca^{2+}/calmodulin-dependent protein kinases in PC12 and A126-B1 cells

Because K^+-stimulated increases in PC12 TH PS are Ca^{2+}-dependent (Table 2), protein kinases that require Ca^{2+} for activation are good candidates for mediating the small 'residual' elevated K^+-stimulated phosphorylation of Ser40 PS in A126-B1 cells. Alternatively, a relative lack of a Ca^{2+}-dependent protein kinase in A126-B1 cells, rather than deficiency in PKA activation, could be responsible for the difference in K^+-stimulated increases in Ser40 PS between PC12 and A126-B1 cells. CAMKII is one of several protein kinases that phosphorylate Ser40 *in vitro.* However, CAMKII protein immunoreactivity was present in and not discernibly different between the PC12 and A126-B1 cells (Fig. 3a), suggesting that the large difference in the magnitude of Ser40 PS between the two cell lines after elevated K^+ treatment was not due to a difference in the abundance of CAMKII protein. As such, CAMKII could mediate the small depolarization-stimulated increase in Ser40 PS seen in A126-B1 cells. Another Ca^{2+}-dependent

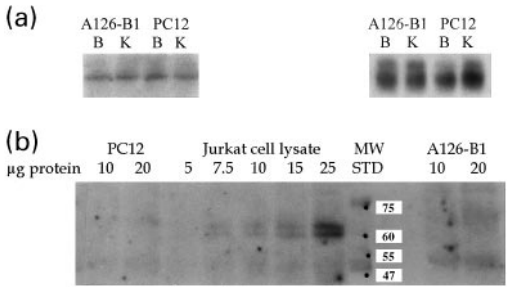

Fig. 3 Blot immunolabeling analysis of CAMKII and CAMKIV in PC12 and A126-B1 cells. (a) After pretreatment, cells were treated for 5 min with buffer (B) or elevated K^+ buffer (K), harvested, and analyzed for CAMKII immunoreactivity (10 μg protein/lane). (a, left) Polyclonal antibody to N-terminal region of CAMKII. (a, right) Monoclonal antibody to rat CAMKIIα. (b) After pretreatment, cells were harvested and analyzed for CAMKIV immunoreactivity (10 and 20 μg protein/lane). Molecular weight standards are given in kDa mass units.

protein kinase that phosphorylates TH *in vitro* is CAMKIV (Miyano *et al.* 1992). However, CAMKIV was not detected in either cell line (Fig. 3b).

A potential involvement of CAMKII in elevated K^+-stimulated Ser19 phosphorylation was tested using a CAMKII inhibitor (KN-93) and its inactive congener (KN-92). Although KN-93 (10 or 15 μM, 20–30 min) inhibited the elevated K^+-stimulated increase in Ser19 PS by 80–100%, KN-92 inhibited the increase in Ser19 PS by nearly 50% under identical conditions (data not shown). The effects of KN-93 and KN-92 were not restricted to Ser19 PS. Elevated K^+-stimulated increases in both Ser31 and Ser40 PS were also inhibited by 50–100% by KN-92 or KN-93. (data not shown). The effects of KN-93 on depolarization-stimulated Ser31 or Ser40 PS were, however, not due to a direct inhibition of ERK- or PKA-mediated phosphorylation of TH, because KN-93 did not reduce the increases in either Ser31 PS or Ser40 PS, associated with NGF (50 ng/mL) or forskolin (0.03 μM) treatment, respectively (data not shown). The non-specific effects of KN-93 on TH PS (Table 2) may reflect its effects on Ca^{2+}-channels, as demonstrated in bovine chromaffin cells (Maurer *et al.* 1996). [In an attempt to create a more specific CAMKII inhibitor, either the autoinhibitory peptide or pseudosubstrate sequence (Ishida *et al.* 1995) was coupled to the Pro50-substituted cell membrane-permeable peptide (Derossi *et al.* 1996). However, concentrations up to 100 μM and pretreatment durations up to 1 h failed to inhibit elevated K^+-stimulated increases in TH PS at Ser19 (data not shown).]

Effects of PD98059 on TH PS and ERK phosphorylation

PD98059 inhibits MEK activation and thereby inhibits the activation of ERK (Alessi *et al.* 1995; Dudley *et al.* 1995). Changes in ERK activity have been correlated directly with changes in Ser31 phosphorylation (Haycock *et al.* 1992).

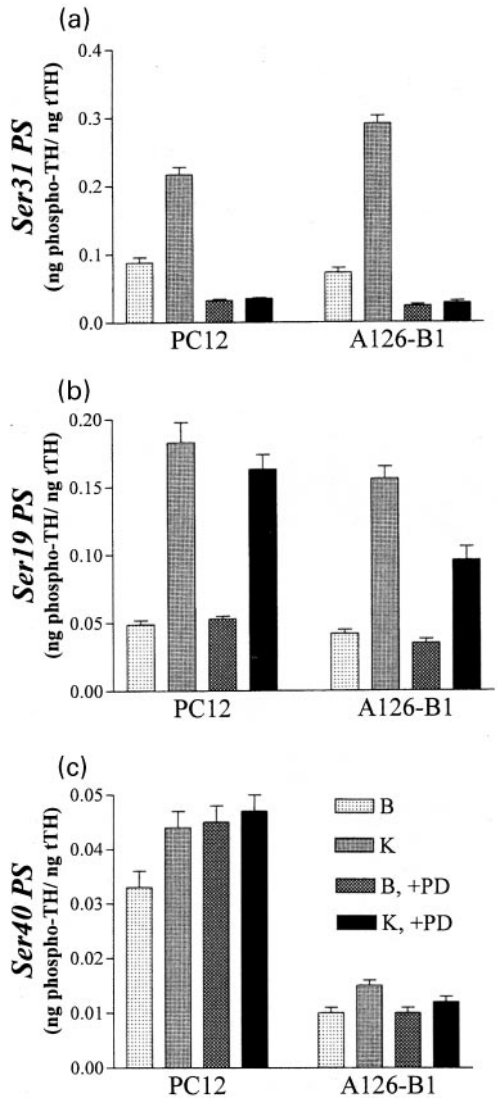

Fig. 4 Effects of the MEK inhibitor PD98059 on basal and elevated K^+-stimulated TH PS in PC12 and A126-B1 cells. Cells were pretreated (30 min) in the absence or presence (+ PD) of 30 μM PD98059. After pretreatment, cells were treated for 5 min with buffer (B) or elevated K^+ buffer (K), and wells and samples were processed for concurrent determination of TH PS and CA biosynthesis as described under Materials and methods. Each bar and associated error bar represents the mean ± SEM ($n = 6$). CA biosynthesis data from the same samples in the experiment shown above are presented in Fig. 6. The data shown are representative of the results from two independent experiments.

Thus, because depolarizing treatments activate ERK in a Ca^{2+}-dependent manner (Pavlovic-Surjancev *et al.* 1992; Frodin *et al.* 1995; Rosenblum *et al.* 2000), PD98059 was used to determine if ERK phosphorylated Ser31 under depolarizing conditions.

Under basal conditions, pretreatment with PD98059 (30 μM, 30 min) reduced Ser31 PS three-fold in both PC12 and A126-B1 cells (Fig. 4a). Ser19 PS was not affected in either cell line (Fig. 4b). PD98059 pretreatment increased Ser40 PS in PC12 cells by 40% and Ser40 PS in A126-B1 cells was unaffected (Fig. 4c).

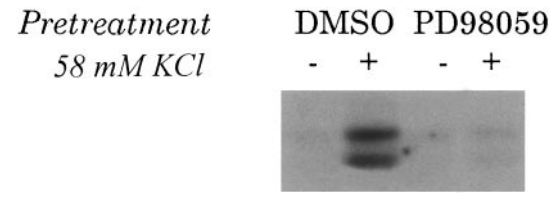

Fig. 5 Effects of PD98059 on basal and elevated K^+-stimulated ERK phosphorylation in PC12 cells. After pretreatment with or without PD98059 (30 μM, 30 min), as indicated in the figure, cells were treated for 5 min with buffer (–) or elevated K^+ buffer (+), harvested, and analyzed for phosphoERK immunoreactivity (17 μg protein/lane). Comparable results were obtained in A126-B1 cells. Assay results reflect observations from a total of 5 experiments in both cell lines.

Pretreatment with PD98059 blocked the elevated K^+-stimulated increase in Ser31 PS in both the PC12 and A126-B1 cells (Fig. 4a). In both cell lines, PD98059 reduced the K^+-stimulated increase in Ser19 PS 25–30% (Fig. 4b). In PD98059-treated PC12 cells, there was no increase in Ser40 PS after K^+-treatment, perhaps as a result of the elevated basal Ser40 PS. However, absolute Ser40 PS from elevated K^+ treatment was unaffected by PD98059 pretreatment. In A126-B1 cells pretreated with PD98059, the apparent decrease in the K^+-stimulated Ser40 PS was not statistically significant. The efficacy of PD98059 in inhibiting elevated K^+-stimulated ERK phosphorylation under the experimental conditions used is illustrated in Fig. 5. Taken together with the observations that ERK phosphorylates Ser31 both *in vitro* and *in situ,* these results indicate that ERK1/ERK2 mediated the elevated K^+-stimulated increase in Ser31 phosphorylation, as previously suggested by Halloran and Vulliet (1994) in bovine chromaffin cells.

Effects of elevated K^+ on CA biosynthesis in PC12 and A126-B1 cells

Treatment with elevated K^+ significantly increased CA biosynthesis 1.5-fold in both cell lines (Fig. 6). However, pretreatment of either cell line with PD98059 prevented the depolarization-induced increase in CA biosynthesis (Fig. 6). These data, in conjunction with the preceding data, suggest that ERK activation and subsequent phosphorylation of Ser31 is responsible for elevated K^+-stimulated increases CA biosynthesis. These data also suggest that elevated K^+-stimulated increases Ser19 PS or Ser40 PS may have little to no influence on CA biosynthesis. In PC12 cells, Ser19 PS and Ser40 PS associated with elevated K^+ treatment were not lowered significantly by pretreatment with PD98059 (Fig. 4); yet, PD98059 effectively prevented

Table 3 Effects of forskolin on Ser40 PS and CA biosynthesis in PC12 cells

Forskolin (μM)	Ser40 PS	pmol $^{14}CO_2$/mg tTH
None	0.028 ± 0.001	1.28 ± 0.16
0.03	0.056 ± 0.002	1.07 ± 0.16
0.30	0.087 ± 0.008	2.19 ± 0.16

PC12 cells were processed as in Table 1 with the exception that after pretreatment, buffer containing the indicated concentrations of forskolin was added. Treatment duration was 5 min. Wells and samples were processed for concurrent determination of TH PS and CA biosynthesis as described under Materials and methods. Control values are presented as mean ± SEM ($n = 6$) and forskolin-treated ($n = 2$) sample data is presented for each sample.

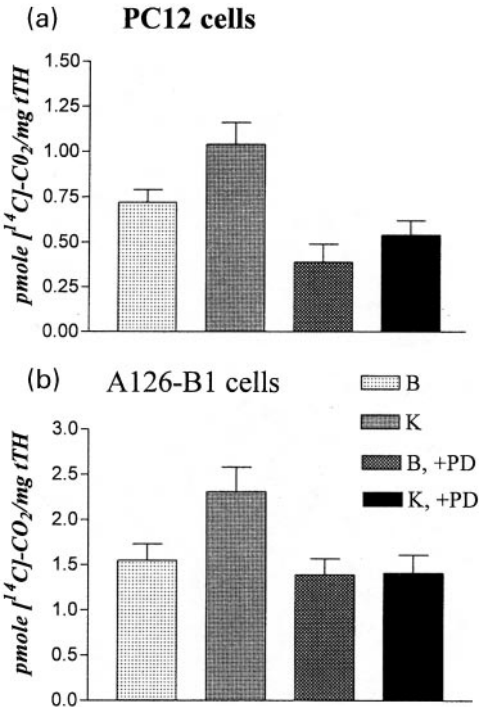

Fig. 6 Effects of PD98059 on basal and elevated K^+-stimulated CA biosynthesis in PC12 and A126-B1 cells. Cells were pretreated (30 min) in the absence or presence (+ PD) of 30 μM PD98059. After pretreatment, cells were treated for 5 min with buffer (B) or elevated K^+ buffer (K), and wells and samples were processed for concurrent determination of TH PS and CA biosynthesis as described under Materials and methods. Each bar and associated error bar represents the mean ± SEM ($n = 6$). TH PS data from the same samples in the experiment shown above are presented in Fig. 4. The data shown are representative of the results from two independent experiments.

any K^+-stimulated increase in CA biosynthesis in both cell lines (Fig. 6). And, even though pretreatment with PD98059 increased Ser40 PS in PC12 cells under basal conditions to an extent similar to that produced by elevated K^+ (Fig. 4c), basal CA biosynthesis was not increased but, in fact, reduced under these conditions (Fig. 6a). Furthermore, K^+-stimulated CA biosynthesis was of the same magnitude in both cell lines, despite a five-fold difference in Ser40 PS between the two cell lines after elevated K^+ treatment (Fig. 4c). In A126-B1 cells, Ser19 PS was increased 2.7-fold by elevated K^+ in the presence of PD98059 while elevated K^+-stimulated CA biosynthesis was completely prevented (Figs 4b and 6b).

Effects of forskolin on CA biosynthesis

The role of Ser40 PS in K^+-stimulated CA biosynthesis in PC12 cells was further evaluated by treating cells with forskolin under conditions (0.03 μM, 5 min) that produced an increase in Ser40 PS comparable to that produced by elevated K^+ treatment (Fig. 2). The goal here was to determine whether this magnitude of change in Ser40 PS would, alone, increase CA biosynthesis in these cells in a manner comparable to elevated K^+ treatment. Treatment of PC12 cells with 0.03 μM forskolin did not increase CA biosynthesis despite a two-fold increase in Ser40 PS (Table 3). However, treatment with a higher concentration of forskolin (0.30 μM) increased Ser40 PS 3-fold and increased CA biosynthesis about 1.7-fold (Table 3). Forskolin treatment at any concentration tested (0.03–0.3 μM) did not significantly affect Ser19 or Ser31 PS (data not shown). These results, taken together with the observation that PD98059 blocks elevated K^+-stimulated increases in CA biosynthesis in PC12 and A126-B1 cells, indicates that the Ser40 PS stimulated by elevated K^+ is not involved in the depolarization-dependent increase in CA biosynthesis.

Effects of NGF on CA biosynthesis

The role of Ser31 PS in K^+-stimulated CA biosynthesis was further evaluated by treating cells with NGF under conditions (50 ng/mL, 5 min) that produced an increase in Ser31 PS comparable to that produced by elevated K^+ treatment. This NGF treatment increased Ser31 PS 2.6-fold in PC12 cells (Fig. 7b, left) and four-fold in A126-B1 cells (data not shown) while CA biosynthesis was increased ~1.5-fold in either cell line (Fig. 7a, left). A longer NGF treatment (10 min) produced a larger increase in Ser31 PS in both cell lines (data not shown), however, the associated CA biosynthesis was not further increased by the longer treatment (data not shown). Thus, although the NGF-stimulation of Ser31 PS and CA biosynthesis supports the conclusion that increasing Ser31 PS to the same magnitude as that produced by elevated K^+ is sufficient to increase CA biosynthesis, the data also indicate that the additional increases in Ser31 PS do not cause further increases in CA biosynthesis.

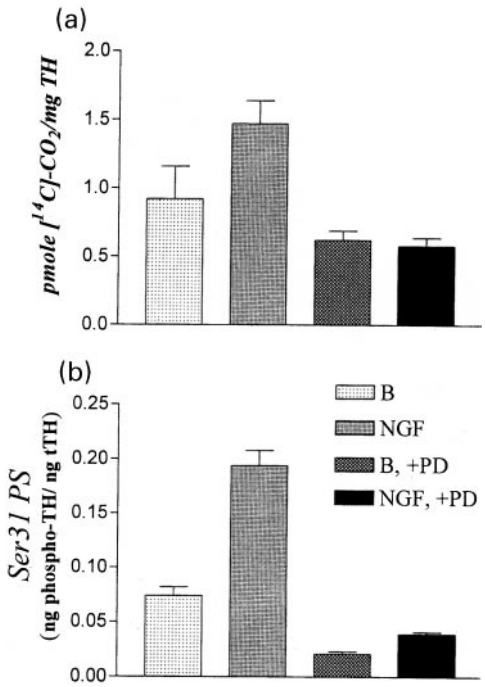

Fig. 7 Effects of NGF on CA biosynthesis and Ser31 PS in PC12 cells. (a) CA biosynthesis under basal conditions (B) or treatment with NGF (NGF, 50 ng/mL, 5 min) after a 30-min pretreatment with or without 30 μM PD98059 (+ PD). (B) Ser31 PS in cells from the same wells as shown in (a). Each bar and associated error bar represents the mean ± SEM ($n = 3$). Similar results were observed in A126-B1 cells (see Results).

The involvement of Ser31 phosphorylation in CA biosynthesis regulation by NGF was also tested by treating cells with NGF after pretreatment with PD98059. PD98059 pretreatment reduced basal Ser31 PS in PC12 cells (Fig. 7b, left; see also Fig. 4a) and in A126-B1 cells (data not shown). Furthermore, PD98059 pretreatment reduced the NGF-stimulated increase in Ser31 PS in PC12 cells (Fig. 7b, right) and in A126-B1 cells (data not shown). Stimulation of phosphoERK immunoreactivity by NGF was also reduced by PD98059 pretreatment in both cell lines (data not shown). Pretreatment with PD98059 completely blocked the NGF-stimulated increase in CA biosynthesis in PC12 cells (Fig. 7a, right) and in A126-B1 cells (data not shown). Taken together, these data support the conclusion that the magnitude of increase in Ser31 PS that is produced by elevated K^+ treatment is necessary and sufficient to increase CA biosynthesis.

Discussion

Depolarization-dependent stimulation of TH phosphorylation, TH activity, and CA biosynthesis has been established to be Ca^{2+}-dependent (Yanagihara *et al.* 1984; Waymire *et al.* 1988; Mitchell *et al.* 1990; Haycock and Wakade 1992). However, the identities of the protein kinases involved in depolarization-stimulated TH phosphorylation at Ser19, Ser31, and Ser40, and the involvement of these sites in regulating CA biosynthesis are not well characterized. Using site- and phosphorylation state-specific antibodies directed toward Ser19, Ser31, or Ser40, we have shown that depolarization increases the PS of all three sites in Ca^{2+}-dependent manner. This confirms and extends studies which used ^{32}P-labeling to study site-specific TH phosphorylation (Waymire *et al.* 1988; Haycock 1990). Thus, the depolarization-dependent acceleration of CA biosynthesis must involve the phosphorylation of one or more of these phosphorylation sites via a protein kinase(s) that requires (directly or indirectly) extracellular Ca^{2+} for activation.

Our approach of assessing the PS of each phosphorylation site using a MEK inhibitor (PD98059, to prevent ERK activation) in both 'normal' PC12 cells and PC12 cells deficient in the ability to activate PKA (A126-B1 cells) permitted evaluation of the role(s) of ERK and PKA in the phosphorylation of the three phosphorylation sites and of the involvement of the three sites in depolarization-stimulated CA biosynthesis. Our results indicate that PKA is predominantly responsible for depolarization-dependent increases in Ser40 phosphorylation but that ERK1/ERK2 activation and Ser31 phosphorylation are responsible for the depolarization-dependent increases in CA biosynthesis.

Protein kinases and site-specific phosphorylation

Ser31

The MEK inhibitor PD98059 decreased basal Ser31 PS and completely prevented the elevated K^+ stimulation of Ser31 PS in both the PC12 and A126-B1 cells. Similarly, PD98059 pretreatment completely prevented the elevated K^+ stimulation of ERK1/ERK2 phosphorylation. Only slight effects of PD98059 on Ser19 PS were observed. PD98059 pretreatment increased basal Ser40 PS in PC12 cells but not in A126-B1 cells, suggesting that there is 'cross-talk' between the ERK and PKA pathways in PC12 cells. Although these data do not distinguish between ERK1 and ERK2 (both of which selectively phosphorylate Ser31 *in vitro*; Haycock *et al.* 1992), they indicate that ERK1/ERK2 mediate the depolarization-dependent phosphorylation of Ser31, as previously suggested for PC12 cells (Haycock *et al.* 1992; Halloran and Vulliet 1994) and bovine chromaffin cells (Thomas *et al.* 1997).

Ser19

CAMKII has been the primary candidate for mediating depolarization-dependent Ser19 phosphorylation *in situ* because it phosphorylates Ser19 *in vitro* and its activation and Ser19 phosphorylation both exhibit similarly rapid increases in response to depolarization (Haycock 1993a; Tsutsui *et al.* 1994). CAMKII (but not CAMKIV) was

present in both the PC12 and A126-B1 cells and elevated K^+ produced substantial increases in Ser19 PS. However, we were unable to show conclusively that CAMKII phosphorylates Ser19 under these conditions. The CAMKII inhibitor KN-93 (as well as its 'inactive' congener, KN-92) proved to be non-specific, as depolarization-stimulated increases in TH PS at all three sites were inhibited by both compounds. In fact, these compounds also inhibited the depolarization-dependent increase in ERK phosphorylation (data not shown). That KN-93 did not affect the selective NGF-dependent increase in Ser31 PS or forskolin-dependent increase in Ser40 PS supports the conclusion of Maurer *et al.* (1996) these compounds probably inhibit Ca^{2+} entry into intact cells. Thus, the protein kinase responsible for depolarization-dependent increases in Ser19 PS remains to be identified.

Ser40

After treatment with elevated K^+, Ser40 PS increased to about 0.05 in PC12 cells compared with 0.01 in A126-B1 cells, in which forskolin failed to elicit an increase in Ser40 PS. This difference in Ser40 PS suggests that PKA is primarily responsible for the depolarization-dependent increase in Ser40 PS. Arguably, the difference in Ser40 PS between the cell lines could be due to differences in their relative abundance of CAMKII (which phosphorylates Ser40 *in vitro*). However, the relative abundance of CAMKII was comparable in both cell lines, strengthening the conclusion that PKA phosphorylates Ser40 under depolarizing conditions. We did detect a consistent and slight increase in Ser40 PS in A126-B1 cells. Although CAMKIV was not detected, perhaps a small amount of Ser40 PS may be mediated by another calcium-dependent protein kinase, such as CAMKII (cf. Thomas *et al.* 1997).

Site-specific phosphorylation and CA biosynthesis

Ser31

Although not previously demonstrated directly, a role for Ser31 phosphorylation in depolarization-stimulated CA biosynthesis can be inferred from previous studies. In AtT-20 cells transfected with TH in which Leu was substituted for either Ser19 (Haycock *et al.* 1998) or Ser40 (Harada *et al.* 1996), elevated K^+-stimulated CA biosynthesis did not differ between cells transfected with TH mutants and wild-type TH. However, the status of Ser31 PS was not determined in these studies, nor were potential interactions between phosphorylation sites (e.g. Ser19 and Ser31 in the Ser40 mutant cells). Our results show that increases in Ser31 PS, and Ser31 alone, are critical to the depolarization-dependent stimulation of CA biosynthesis. Elevated K^+ treatment resulted in an increase in phosphorylated (hence, activated) ERK, which phosphorylated Ser31 and in turn increased CA biosynthesis. Moreover, all of these effects were blocked by PD90859. That Ser31 phosphorylation is necessary and sufficient for the depolarization-dependent increases in CA biosynthesis is further supported by the observations that PD98059 blocked increases in both Ser31 PS and CA biosynthesis in PC12 and A126-B1 cells and that NGF treatment (under conditions which mimicked the effects of elevated K^+ on Ser31 PS) increased CA biosynthesis to the same extent as elevated K^+ (~50% increase) in both cell lines.

Ser19

A potential involvement of Ser19 phosphorylation in depolarization-stimulated CA biosynthesis in intact cells has been inferred from the observation that the CAMKII inhibitors KN-93 and KN-62 inhibit depolarization-stimulated CA biosynthesis (Sumi *et al.* 1991; Marley and Thomson 1996). However, our data shows that KN-93 inhibited the depolarization-dependent increases in phosphorylation at each of the TH sites. Thus, the inhibition of depolarization-stimulated Ser31 phosphorylation by KN-93 pretreatment that we observed is probably the reason behind the previously reported inhibitory effects of KN-93 on depolarization-stimulated TH activity and CA biosynthesis. In agreement with Marley and Thomson (1996), who reported that KN-92 (the inactive congener) inhibited depolarization-stimulated TH activity, we found that KN-92 inhibited depolarization-stimulated ERK and Ser31 phosphorylation to an extent similar to that produced by KN-93. Our data also show that even though PD90859 pretreatment blocked the depolarization-dependent increases in CA, Ser19 PS was still increased about three-fold in both cell lines. From these data, and as supported by previous studies using alternative approaches (Haycock *et al.* 1998; Lindgren *et al.* 2000), we conclude that neither Ser19 phosphorylation nor CAMKII phosphorylation of TH is directly involved in depolarization-stimulated CA biosynthesis.

Ser40

The disparity in Ser40 PS but not in the regulation of depolarization-stimulated CA biosynthesis between PC12 and A126-B1 cells suggests that Ser40 phosphorylation plays little or no role in depolarization-dependent CA biosynthesis. Yet, PKA-mediated phosphorylation of Ser40 could play a role in regulating CA biosynthesis under other conditions. Several reports have shown a direct relationship of Ser40 phosphorylation with regulating TH activity or CA biosynthesis *in situ* and *in vivo* (Waymire *et al.* 1991; Lew *et al.* 1998; Lindgren *et al.* 2000, 2001). The apparent lack of relationship between Ser40 phosphorylation and CA biosynthesis under depolarizing conditions may be that an inadequate level of Ser40 PS is achieved under depolarizing conditions in PC12 cells. For example, whereas forskolin at a concentration that increased Ser40 PS to 0.05 (i.e. similar to that produced by elevated K^+) did not stimulate CA

biosynthesis, a 10-fold higher forskolin concentration increased Ser40 PS to 0.09 and increased CA biosynthesis 1.7-fold. Therefore, a certain threshold of PS at Ser40 may have to be reached before an effect on CA biosynthesis occurs. Agents that affect PKA activity such as neuropeptides, NMDA agonists, or D2 autoreceptor ligands (Waymire *et al.* 1991; Lindgren *et al.* 2000, 2001), alone or in combination with depolarization, may increase Ser40 PS sufficiently to increase CA biosynthesis.

Implications for CA biosynthesis *in vivo*

The present evidence that thresholds of PS must be exceeded in order to influence CA biosynthesis may have implications for CA function in the CNS. Ser40 PS in the striatum, nucleus accumbens, substantia nigra, and ventral tegmental area (VTA) is ~0.03 (Salvatore *et al.* 2000), similar to that in PC12 cells. Ser31 PS in the substantia nigra and ventral tegmental area (~0.10) and Ser19 PS in the striatum (~0.10) are also similar to those in PC12 cells. We reported the effects of haloperidol on TH PS *in vivo* in these dopaminergic brain regions and compared these results with previous work in which the effects of haloperidol on TH activity and CA biosynthesis had been reported. Haloperidol has been reported to have far less influence on TH activity and CA biosynthesis in cell body regions than in terminal field regions. After acute haloperidol, Ser40 PS was increased to a greater extent in the terminal field than in the cell body regions whereas Ser31 PS was increased to the same extent in both regions. The conclusion from these observations was that Ser40 PS, and not Ser31 PS, was associated with the regional differences in the effects of haloperidol on CA biosynthesis. However, the actual difference in Ser31 PS between the terminal field and cell body regions associated with acute haloperidol was about 0.40 (0.50–0.60 PS in the terminal field regions versus 0.15–0.20 PS in the cell body regions). The findings of the present PC12 study emphasize that it is the absolute level of PS, and not the percent of control increase in PS, that influences CA biosynthesis. Thus the large difference in Ser31 PS between the terminal field and cell body regions could be a major factor in the regional differences in CA biosynthesis observed after acute haloperidol.

Our present results indicate that ERK-mediated phosphorylation of Ser31, but not PKA-mediated phosphorylation of Ser40, is critical for replenishing CA not only secreted from PC12 cells under depolarizing conditions but, as supported in the preceding paragraph, lost during neuronal activity in dopaminergic brain regions. These findings, coupled with previous biochemical investigations into the etiology of neurological diseases involving CA systems make it plausible that Ser31 phosphorylation of TH plays a role in these diseases. For example, chronic administration of morphine or cocaine increases phospho-ERK immunoreactivity in the VTA (Berhow *et al.* 1996), suggesting that dopamine biosynthesis may be elevated in this region. In fact, amphetamine-sensitive rats have an increase in striatal NGF-related neurotrophin expression and TH activity (Solbrig *et al.* 2000). In addition, agents that affect PKA activity can influence cocaine self-administration (Self *et al.* 1998), and elevated impulse activity in midbrain dopamine neurons is associated with increased vulnerability to cocaine self-administration (Marinelli and White 2000). Thus, that both ERK and PKA are activated by depolarization underscores the importance of considering the regulation of both of Ser31 and Ser40 phosphorylation in such behavioral paradigms.

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