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A dual role for the cAMP-dependent protein kinase in tyrosine hydroxylase gene expression

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ABSTRACT **Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine, the first and rate-limiting step in catecholamine biosynthesis. The cAMP-dependent protein kinase (PKA) phosphorylates and activates the TH enzyme and is thought to mediate transcriptional induction of the TH gene. To better understand the functional role of PKA in TH gene regulation, we studied TH gene expression at the transcriptional, translational, and post-translational levels in several PKA-deficient cell lines derived from rat PC12 pheochromocytoma cells. Strikingly, all PKA-deficient cell lines analyzed in this study showed substantial deficits in basal TH expression as measured by TH enzymatic activity, level of TH immunoreactivity, TH protein level, and steady-state mRNA level. Interestingly, the steady-state level of mRNA correlated well with levels of TH activity, immunoreactivity, and protein. In addition, PKA-deficient cell lines lacked transcriptional induction of the TH gene following treatment with dibutyryl cAMP. Cotransfection of PKA-deficient cells with an expression plasmid for the catalytic subunit of PKA fully reversed transcriptional defect, as indicated by robust transcriptional induction of a reporter construct containing 2400 bp of TH upstream sequence in all PC12 cells tested. These data indicate that the PKA system regulates both the basal and the cAMP-inducible expression of the TH gene primarily at the transcriptional level in PC12 cells.**

The nervous system performs important adaptive processes through activation of various postsynaptic receptors and their associated signal transduction pathways. These processes can elicit either acute changes in the function of proteins—for instance, enzyme activities—by covalent modification of preexisting molecules or delayed changes in levels of protein by altering gene expression. The regulation of neurotransmitter-biosynthesizing-enzyme genes in particular is thought to play a pivotal role, since the resultant change in the amount of available neurotransmitter molecules can trigger substantial variations in neuronal activity.

Tyrosine hydroxylase [TH; L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] catalyzes the conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-dopa), which is the first and rate-limiting step in the biosynthesis of the catecholamine neurotransmitters (1). A variety of trans-synaptic and hormonal stimuli are known to produce either acute increases in TH activity via phosphorylation of the preexisting enzyme proteins (refs. 2 and 3; for review see ref. 4) or delayed increases in TH (5–7). The latter responses correlate with increases in TH mRNA and enzyme molecules and are blocked by inhibitors of RNA and protein synthesis, indicating that transcriptional regulation of the TH gene is directly involved (8, 9).

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Different signaling pathways are likely to function in post-translational and transcriptional responses. For example, cAMP-dependent protein kinase (PKA; ATP:protein phosphotransferase, EC 2.7.1.37) was shown to enhance the activity of TH, via phosphorylation of the TH protein, by lowering the K_m value for its cofactor, tetrahydrobiopterin (2, 3). Activation of PKA also appears to induce transcription of the TH gene (8, 9). The 5′ flanking sequence of the TH gene contains several putative cis-acting motifs [e.g., AP1 and AP2 binding sites and a cAMP response element (CRE) (10)] potentially capable of mediating the transcriptional response to the cAMP-mediated PKA signaling pathway. We have conducted extensive deletional and mutational analyses of 5′ upstream sequence of the TH gene in rat pheochromocytoma PC12 and human neuroblastoma SK-N-BE(2)C cells. Our data strongly suggest that the consensus CRE (5′-TGACGTCA-3′) at bp −38 to −45 upstream of the TH gene exerts essential roles for the basal as well as cAMP-induced transcription of that gene (11). This CRE presumably interacts with the 43-kDa CRE-binding protein (CREB) that, when phosphorylated by PKA, activates the transcription of genes containing CREs (12, 13). In contrast, earlier studies suggested that the AP1 site and its adjacent region are crucial for transcription and that the CRE is not important for TH gene transcription (14–16). The reason for this apparent discrepancy is unclear.

Here we report experiments on the role of PKA in TH gene transcription in intact cells. We used PC12 cell lines which had been rendered PKA-deficient either by genetic manipulation (17) or by chemical mutagenesis (18). All mutant cell lines tested exhibited considerable loss of basal and inducible expression of the TH gene. Furthermore, transient cotransfection analyses confirmed that the PKA system participates in the transcriptional control of the TH gene in PC12 cells.

MATERIALS AND METHODS

Cell Culture and Immunocytochemistry. PC12 cells and the PKA-deficient cell lines AB.11, 123.7, and A126-1B2 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (dialyzed) and 5% donor horse serum (dialyzed) without heat inactivation. For some experiments, adenosine deaminase (Sigma) was included in the medium at 0.8 unit/ml. Cells were maintained in a humidified 10% CO_2 environment at 37°C. For immunocytochemical staining, the cells were fixed with formaldehyde and incubated overnight with a specific polyclonal antiserum to TH (diluted 1:25,000), which was prepared as described (6). Subsequent steps were performed with the Vectastain Elite kit (Vector Laboratories).

Abbreviations: PKA, cAMP-dependent protein kinase; TH, tyrosine hydroxylase; CRE, cAMP response element; CREB, CRE-binding protein; PKA_c, catalytic subunit of PKA; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; Bt_2cAMP, dibutyryl cAMP.

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TH Enzyme Assay and Western Blot Analysis. Cells were harvested at 10^5 per cm^2 and washed with phosphate-buffered saline. The cell pellet was homogenized in 5 mM potassium phosphate buffer, pH 7.0/0.2% Triton X-100 and centrifuged at 16,000 × *g* for 10 min. The resultant supernatant was used for enzyme assay, protein assay, and Western blot analysis. TH enzyme activity was measured by using 0.2 mM [^{14}C(U)]tyrosine and 1 mM 6-methyltetrahydropterine as described (19). Protein concentration was determined by the Lowry method. Western blotting was performed according to Burnette (20).

Poly(A)$^+$ RNA Extraction and Northern Blot Hybridization. Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose affinity column chromatography (21). Poly(A)$^+$ RNA (1 μg per lane) was electrophoresed in a 1% agarose gel, transferred to a nylon membrane (Amersham), and hybridized with a rat TH cDNA probe labeled with [α-^{32}P]dCTP to a specific activity of 1–2 × 10^9 dpm/μg by random hexamer priming. For rehybridization, the previous signal was removed by thoroughly washing the membrane in 80–90°C 1.5 mM NaCl/0.15 mM sodium citrate, pH 7/0.01% SDS (five to eight times) until no signal was detected after overnight exposure. Blots were autoradiographed on an intensifying screen for 4–12 hr at −70°C. Steady-state levels of mRNA were quantitated with a PhosphorImager and IMAGEQUANT software (Molecular Dynamics) and were normalized to those of the control gene, α-tubulin.

Transient Transfection and Plasmids. A reporter plasmid, pTH2400CAT (11), that includes the upstream sequence from −2400 to +27 of the rat TH gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene was used in cotransfection experiments. Transfection of reporter plasmids into PC12 cells was performed by the calcium phosphate coprecipitation method (22). PKA-deficient cells were transfected with slightly higher efficiency than wild-type PC12 cells. To control for this difference in transfection efficiency, a plasmid containing the Rous sarcoma virus (RSV) promoter fused to the CAT reporter gene (pRSVcat; ref. 23) was transfected into parallel cultures as a positive control. A RSV–β-galactosidase plasmid was included as an internal control to normalize for the different transfection efficiencies from dish to dish (24). The expression plasmid for the catalytic subunit of PKA (PKA_c) has been described (13). The plasmids utilized in this study have been prepared at least twice independently and confirmed to produce identical results.

RESULTS

A straightforward approach to investigating the roles of PKA in TH gene regulation is to utilize PKA-deficient cell lines. We analyzed TH gene expression in three PKA-deficient subclones of PC12: A126-1B2 (18), isolated on the basis of resistance to dibutyryl cAMP (Bt_2cAMP) and cholera toxin after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis, and two cell lines, 123.7 and AB.11, which stably express mutant regulatory subunits (RI) of PKA. The RI subunits of 123.7 cells contain two point mutations in the site B cAMP binding site, while those of AB.11 cells contain mutations in both site A and site B (17). Each of these cell lines contains only 10–20% of PKA activity compared with wild-type PC12 cells, but levels of protein kinase C and Ca^{2+}/calmodulin-dependent kinase are not changed (17, 25).

TH Enzyme Activity and Protein Amount Are Altered in PKA-Deficient Cells. AB.11 and 123.7 cells exhibited, respectively, 41% and 45% of the TH activity of the wild-type PC12 cells (Table 1). TH activity was almost nondetectable in the A126-1B2 cell line. To examine the possibility that deficient TH activity in the PKA-deficient cells reflects altered phosphorylation of existing TH molecules, we performed a kinetic analysis of the TH activity from these cells. The K_m value for the cofactor, 6-methyltetrahydrobiopterin, was not changed for TH enzyme obtained from PKA-deficient cells (data not shown). These results thus failed to support the hypothesis that altered TH phosphorylation fully accounts for the diminished TH activity of the PKA-deficient cells. We then performed experiments to determine whether the PKA-deficient cells contained low levels of TH protein. First, we compared the relative amount of TH immunoreactivity in the absence (data not shown) or presence of Bt_2cAMP (Fig. 1). All mutant cells had lower levels of TH immunoreactivity than wild-type PC12 cells, indicating that the reduced TH activity in the PKA-deficient cells reflects a decrease in the number of TH molecules. As previously reported (17), only wild-type PC12 cells formed abundant neurites following treatment with Bt_2cAMP. Western blot analysis confirmed that the amounts of TH protein were considerably decreased in the PKA-deficient cells (Fig. 2).

Table 1. TH enzyme activity in wild-type (WT) and PKA-deficient PC12 cell lines

Cell line	TH specific activity: nmol/mg of protein	% of WT
PC12 WT	59.1 ± 6.6 (8)	100
AB.11	24.3 ± 2.3 (4)*	41
123.7	26.4 ± 4.1 (6)*	45
A126-1B2	0.1 ± 0.1 (6)**	<1

TH specific activity is shown as mean ± SEM for 10-min incubations at 30°C. Numbers in parentheses represent the number of culture dishes used separately for the assay. *, $P < 0.01$; **, $P < 0.001$.

Both Basal and cAMP-Mediated Inducible Transcription Are Altered in PKA-Deficient Cells. Our observation that PKA-deficient cells contained less TH protein prompted us to examine the steady-state mRNA levels by Northern blot analysis. All PKA-deficient cells contained less TH mRNA than the wild-type cells (Fig. 3), consistent with their levels of TH activity and protein. In contrast, the mRNA level of α-tubulin mRNA, which was used as an internal control, was not altered at all in any of the cell lines (Fig. 3). The regulation of TH gene transcription was further investigated by adding extracellular Bt_2cAMP to these cells in the presence of adenosine deaminase to prevent autoregulation by secreted adenosine (26). This treatment increased TH mRNA about 2-fold in PC12 cells but did not induce TH message in AB.11 and 123.7 cells (Fig. 4 and data not shown). Similar observations were made when adenosine deaminase was omitted from the medium (data not shown). These data support the idea that the PKA system is essential not only for maintaining the uninduced, basal level of transcription but also for the cAMP-mediated transcriptional induction of the TH gene.

Transient-Transfection Analyses Demonstrate That PKA Is an Important Determinant of TH Gene Transcription. Since PKA deficiency diminished TH expression, it was likely that an excess of PKA would increase the transcription of the TH gene. To test this hypothesis, we used a reporter plasmid, TH2400CAT (11), in which the upstream sequence of the rat TH gene, bp −2400 to +27, was fused to the CAT reporter gene. Cotransfection of a constant amount of TH2400CAT with increasing amounts of an expression vector for PKA_c resulted in a dose-dependent increase in CAT expression in parental PC12 cells (Fig. 5). In contrast, the RSV–CAT plasmid (pRSVcat) was not induced by cotransfection with PKA_c (Fig. 5). To find out whether excess PKA_c could reverse the decrease in TH gene transcription, we also performed cotransfection experiments in PKA-deficient cell lines. Under basal conditions, AB.11 and 123.7 cells pro-

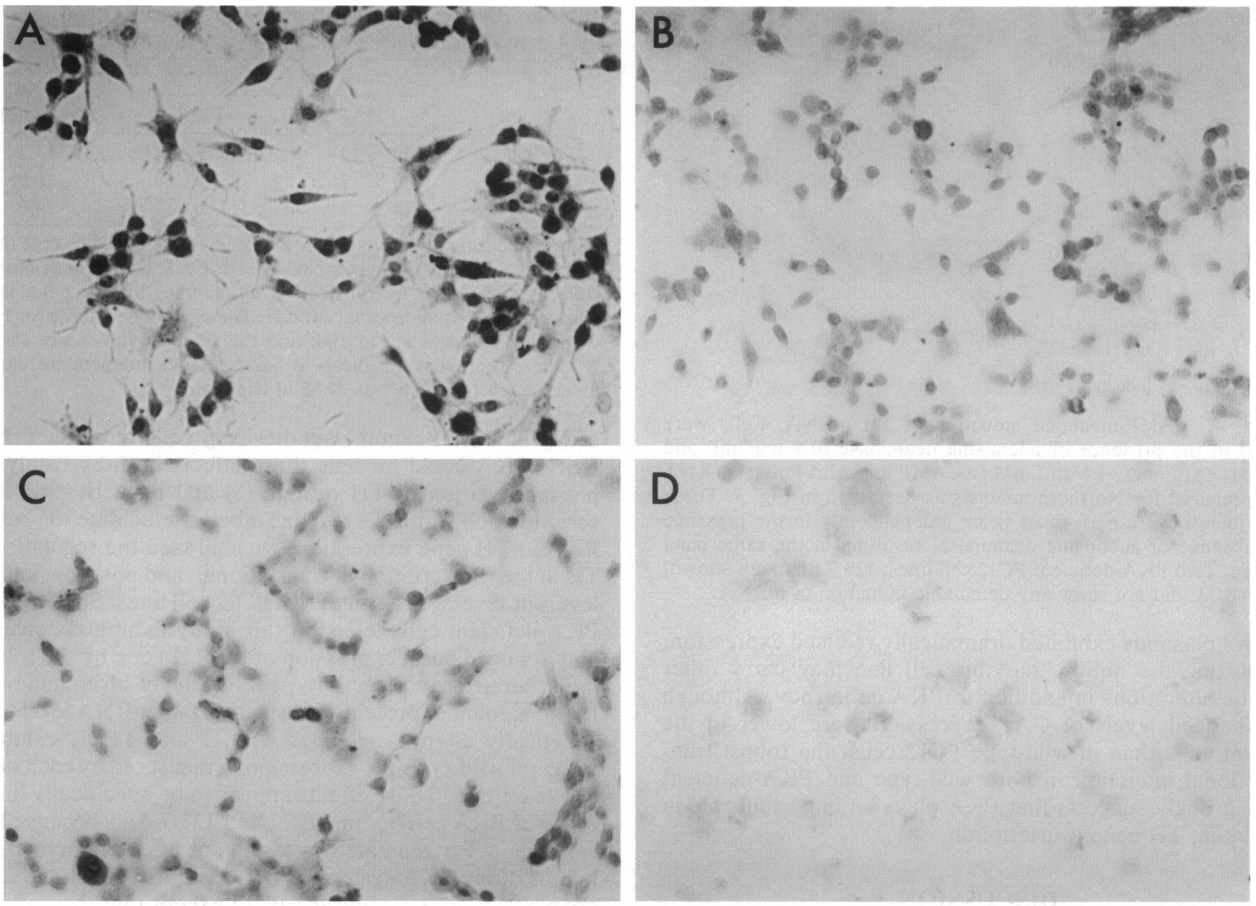

FIG. 1. TH immunoreactivity of wild-type and PKA-deficient PC12 cells following treatment with Bt_2cAMP. Wild-type (*A*), AB.11 (*B*), 123.7 (*C*), and A126-1B2 (*D*) cells were grown in plastic culture chambers, treated with 1 mM Bt_2cAMP overnight, and then processed for immunochemistry.

duced 30–40% as much CAT activity as wild-type PC12 cells (Table 2), an observation that is consistent with their reduced expression of the endogenous TH gene. Thus, the transient-transfection assay represented the transcriptional efficacy of the TH gene in each cell line. Cotransfection of mutant lines with PKA_c expression plasmid stimulated transcription 15- to 19-fold, overcoming the PKA-dependent transcriptional defect in PKA-deficient cells. Similar results were obtained in the chemically mutated A126-1B2 cells (data not shown). However, a direct comparison with other cell lines was difficult because the RSV–CAT and RSV–β-galactosidase

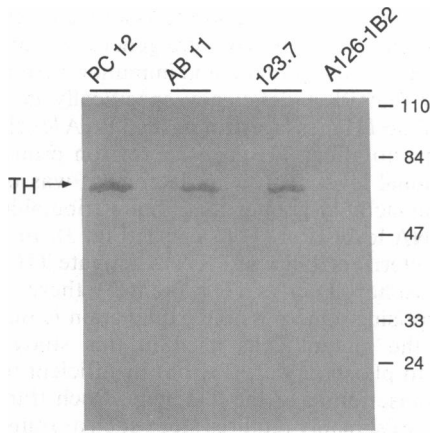

FIG. 2. Western blot analysis of TH from wild-type and PKA-deficient PC12 cells. In each lane, 50 μg of protein prepared from each cell line was electrophoresed in an SDS/12% polyacrylamide slab gel and immunoblotted with TH antiserum. Intensities of the TH band at M_r 64,000 (arrow) correspond well with TH enzyme activities shown in Table 1. Positions of marker proteins of known molecular weight (Bio-Rad) are shown at right ($M_r \times 10^{-3}$).

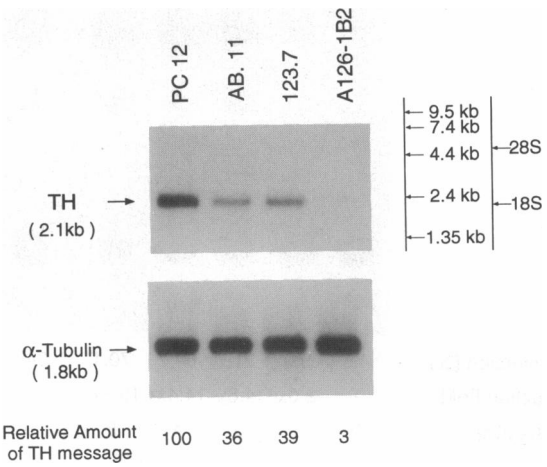

FIG. 3. Basal levels of TH mRNA by Northern analysis. Poly(A)$^+$ RNA (1 μg per lane) was electrophoresed in a 1% agarose gel containing 2 M formaldehyde. The same blot was used consecutively for hybridization with TH and α-tubulin probes. All bands were quantitated with a PhosphorImager analyzer (Molecular Dynamics) and normalized to calculate relative amount of TH message. Three separate experiments showed identical patterns with <5% absolute variability in the ratios of the message signals. Locations of RNA size standards (BRL) as well as 18S and 28S rRNA are indicated at right of the upper autoradiogram.

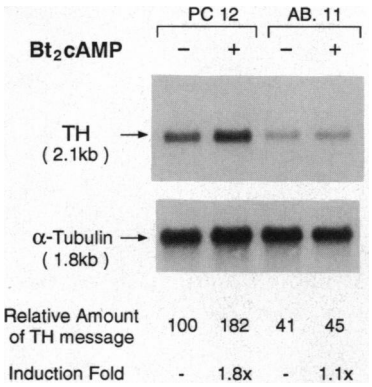

FIG. 4. cAMP-mediated induction of TH mRNA. Cells were grown in the presence of adenosine deaminase (0.8 unit/ml) and treated (+) or not (−) with 1 mM Bt_2cAMP for 12 hr. Poly(A)$^+$ RNA was prepared for Northern analysis as described in Fig. 3. These experiments were performed twice independently in the presence and absence of adenosine deaminase, resulting in the same band pattern. Two PKA-deficient PC12 cell lines, 123.7 (data not shown) and AB.11, did not show any detectable induction of mRNA.

control plasmids exhibited dramatically reduced expression, supporting the notion that this cell line may carry other genetic alterations in addition to PKA deficiency. Although the induced levels of CAT expression were lower in the mutant cells than in wild-type PC12 cells, the robust transcriptional induction in both wild-type and PKA-deficient cells strongly suggests that PKA plays an important role in regulating TH gene transcription.

DISCUSSION

cAMP, a well-characterized second messenger, serves many important cellular functions. The PKA signaling pathway appears to mediate the cAMP response in eukaryotic cells (27). As reviewed elsewhere (4, 28), increases in intracellular

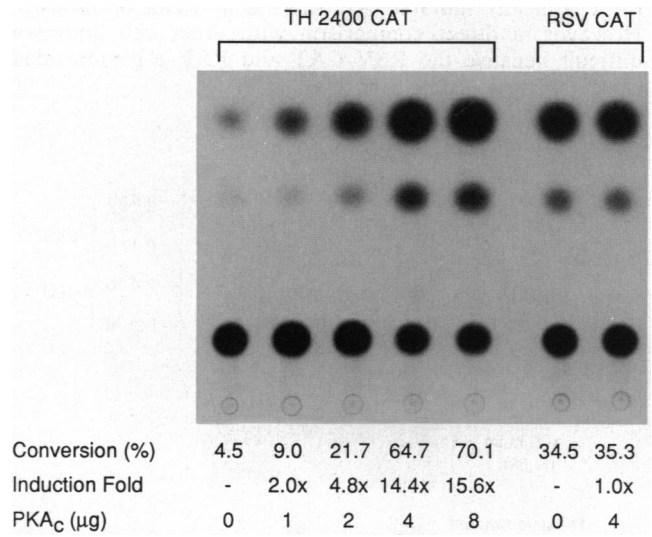

FIG. 5. Cotransfection analysis in PC12 cells. Reporter plasmids are indicated above the autoradiogram. Each 10-cm tissue culture dish received a total of 25 μg of DNA comprising 8 μg of reporter plasmid, 2 μg of RSV–β-galactosidase plasmid, and PKA_c expression plasmid as indicated; pUC19 was used as inert DNA. This transfection assay was repeated three times in triplicate, resulting in similar patterns. An autoradiogram of a representative thin-layer chromatogram is shown. Percent conversion of chloramphenicol into the butyrylated forms (upper two spots) was determined by cutting out each spot for scintillation counting and is shown below the autoradiogram.

Table 2. Cotransfection analysis of wild-type PC12 and PKA-deficient cell lines with PKA_c expression plasmid

Cell line	Normalized CAT activity		Fold induction
	− PKA_c	+ PKA_c	
PC12	100 ± 14	1320 ± 104	13.2
AB.11	39 ± 7	737 ± 58	18.9
123.7	35 ± 3	511 ± 37	14.6

Transfection conditions were the same as for Fig. 5 except that 4 μg of PKA_c expression vector was used. CAT activity was normalized for differences in transfection efficiencies by using β-galactosidase activity as an internal control. The normalized value for PC12 without PKA_c was set at 100, and the relative values are shown. Numbers represent the mean ± SEM of two independent experiments, each of which was done in triplicate.

cAMP in catecholamine-synthesizing cells increase TH activity. PKA could mediate these effects both by catalyzing phosphorylation of TH protein (3) and by activating transcription of TH mRNA (8, 9). To better elucidate the role of PKA in TH gene expression, we analyzed the regulation of TH at the transcriptional, translational, and posttranslational levels in several PKA-deficient PC12 cell lines. Strikingly, all PKA-deficient cells tested in this study exhibited considerably reduced basal expression of the TH gene by the following criteria: enzyme activity, cellular level of immunoreactivity, amount of protein, and steady-state mRNA level. Two genetically altered cell lines, AB.11 and 123.7, exhibited <50% of wild-type TH expression as measured by each of the above criteria. Those effects appear to be specifically linked to lower PKA activity in AB.11 and 123.7 cells because both cell lines were generated by using specific genetic manipulations of the PKA system (17). Thus, both AB.11 and 123.7 are isogenic to the wild-type PC12 except that they overexpress a mutant regulatory subunit of PKA that does not dissociate from the catalytic subunit in response to cAMP (29). Moreover, levels of protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase are not altered in AB.11 and 123.7 cells (17, 25). Differences in those other signaling pathways are thus unlikely to explain the present findings. In contrast to AB.11 and 123.7 cells, TH expression was almost abolished in A126-1B2 cells. Since the genetic defects are not fully characterized in this chemically mutagenized cell line (18), it is plausible that there exist additional mutations other than PKA deficiency which affect transcription of the TH gene in A126-1B2.

Comparison of wild-type and PKA-deficient cells revealed that relative levels of mRNA were generally proportional to those of TH activity, protein, and immunoreactivity. Since it is unlikely that PKA deficiency specifically decreases the stability of the TH message, diminished PKA levels in mutant cells appear to affect TH gene expression primarily at the transcriptional level. Thus, no decrement was observed in TH enzyme activity greater than that attributable to diminished mRNA levels for TH (Table 1; Fig. 3). In light of the well-characterized ability of PKA to activate TH by catalyzing the phosphorylation of TH protein (3), these results were rather surprising. One possible explanation is that the PKA levels in the mutant cells used in this study were still sufficient to phosphorylate TH but insufficient to stimulate optimal transcription of the TH gene. Such transcriptional activation presumably requires steps such as translocation of PKA into the nucleus and phosphorylation of the target transcription factor, whereas these steps are unnecessary for phosphorylation of cytoplasmic TH molecules. Alternatively, we might not have detected as acute and dynamic a process as activation of TH by phosphorylation, because our experiments were designed to detect long-term changes in steady-state levels of TH expression.

In response to treatment with an analog of cAMP, only wild-type PC12 cells exhibited induction of TH mRNA (Fig. 4). Thus, PKA seems to be critical not only for basal expression but also for transcriptional induction of the TH gene in response to increases in intracellular cAMP. A similar conclusion regarding the role of PKA in TH gene induction was drawn from a study which showed that treatment with vasoactive intestinal polypeptide or forskolin increased TH transcription in wild-type PC12 cells but not in A126-1B2 cells (30). Moreover, overexpression of PKA_c specifically increased the transcriptional activity of TH upstream sequence in both PC12 and PKA-deficient cells (Fig. 5) in our transient-transfection assay. Using elegant cotransfection analyses, Mellon *et al.* (31) demonstrated that cAMP can regulate, via PKA, basal as well as induced expression of the α-subunit gene of the human glycoprotein hormones, which contains the CRE. Our data suggest a similar role for PKA in the regulation of TH gene expression. Recent studies indicated coordinate regulation of TH expression and the cAMP system in locus coeruleus by chronic stress, catecholamine depletion, and various drug and hormone treatments (32, 33), suggesting the involvement of PKA in TH gene regulation in this region of the brain.

We propose that the PKA system plays two important roles in the regulation of TH gene expression, as well as a role in modifying the activity of the protein. First, PKA is crucial for basal transcription and expression of the TH gene in TH-expressing cells, since PKA deficiency resulted in significant loss of TH expression in all mutant PC12 cells tested. In this context, it is important that the CRE may have a dual role as a basal promoter element and an inducible enhancer for other cAMP-responsive genes (34). Our recent analyses confirmed the essential dual role of the CRE in the transcriptional regulation of the TH gene (11). Second, PKA probably mediates transcriptional induction of the TH gene in response to increases in cAMP brought about by a variety of different signals (Fig. 4). Induction of the TH gene by PKA might thus be an important mediator of alterations of neuronal activity in response to environmental stimuli (7). Additionally, PKA can exert important acute regulation of TH activity in response to many stimuli by phosphorylation of the enzyme molecule (3).

Among the growing family of transcription factors that can bind to the CRE-like motifs (35), one candidate molecule capable of mediating the effect of PKA on TH transcription is CREB (11, 12). It has been suggested that a common trans-acting element might coregulate several neuronally expressed genes such as those encoding somatostatin, TH, vasoactive intestinal polypeptide, and proenkephalin (36). Phosphorylation of CREB is essential for transcriptional activation of cAMP-regulated target genes (12, 13). Thus, PKA might control the target genes by directly regulating the phosphorylation of CREB. In addition, phosphorylated CREB can turn on transcription of the gene for at least one other transcriptional activator, c-Fos (37), which in turn can contribute to activation of the TH gene. Indeed, the 5′ flanking sequence of the TH gene contains both relevant cis-acting motifs: CRE and AP1. Thus, it is conceivable that these two mechanisms work cooperatively for the regulation of TH gene transcription in response to different stimuli.

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