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Identification of protein phosphatase 2A as the major tyrosine hydroxylase phosphatase in adrenal medulla and corpus striatum: evidence from the effects of okadaic acid

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(i) The major sites on bovine adrenal tyrosine hydroxylase (TH) phosphorylated by calmodulin-dependent multiprotein kinase (CaM-MPK) and cyclic AMP-dependent protein kinase were shown to be Ser-19 and Ser-40, respectively, while Ser-40 was also phosphorylated slowly by CaM-MPK. (ii) Type 2A and type 2C phosphatases accounted for ≈90% and ≈10% of TH phosphatase activity, respectively, in extracts of adrenal medulla and corpus striatum assayed at near physiological free Mg^{2+}(1 mM), while type 1 and type 2B phosphatases had negligible activity towards TH. (iii) Incubation of adrenal chromaffin cells with okadaic acid increased TH phosphorylation by 206% and activity by 77%, establishing that type 2A phosphatases play a major role in regulating TH in vivo.

Tyrosine hydroxylase; Protein phosphatase; cyclic AMP; Calmodulin; Protein kinase; Okadaic acid; Protein sequencing

1. INTRODUCTION

Tyrosine 3-monooxygenase (tyrosine hydroxylase (TH); EC 1.14.16.2), the rate-limiting enzyme in catecholamine biosynthesis [1], is mainly located in the adrenal medulla and brain, where it is enriched in the striatum. The enzyme is phosphorylated and activated by a variety of protein kinases in vitro, including cyclic AMP-dependent protein kinase (A-kinase), calmodulin-dependent multiprotein kinase (CaM-MPK), protein kinase C, and less well defined kinases associated with purified TH (reviewed in [2]). Three phosphorylation sites have been identified in TH from rat pheochromocytoma (an adrenal tumour). Serine 8 is labelled by a protein kinase that contaminates purified TH, Ser-19 by CaM-MPK and Ser-40 by A-kinase [3]. Ser-40 is also phosphorylated by CaM-MPK, although at a lower rate than Ser-19. TH is multiply phosphorylated in adrenal chromaffin cells [4,5], brain [6] and PC12 pheochromocytoma cells [7] and phosphorylation increases in response to a variety of agonists, such as acetylcholine, K^+ depolarisation, cyclic AMP analogues or phorbol esters (reviewed in [2]). However, the precise sites phosphorylated in vivo have still to be defined.

Very little is known about the protein phosphatases that dephosphorylate TH. Yamauchi and Fujisawa [8] found that TH activity in cytosol of bovine adrenal medulla was inhibited by sodium phosphate and fluoride and stimulated by Mg^{2+}, while Nelson and Kaufman [9] resolved three peaks of TH phosphatase activity after chromatographing extracts of rat caudate nucleus on DEAE cellulose. A peak eluting at 0.3 M NaCl was the only one which dephosphorylated TH

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Abbreviations: TH, tyrosine hydroxylase; A-kinase, cyclic AMP-dependent protein kinase; CaM-MPK, calmodulin-dependent multiprotein kinase

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phosphorylated by A-kinase and the major one which dephosphorylated TH labelled by CaM-MPK. Two further peaks with activity towards TH phosphorylated by CaM-MPK were eluted at lower NaCl and reported to be inhibited by 1 mM Ca^{2+} [9].

Large quantities of homogeneous tyrosine hydroxylase from bovine adrenal medulla have recently become available [10], sufficient for making the ^{32}P-labelled enzyme needed to identify the sites of phosphorylation and to characterise TH phosphatases. In this paper, we identify the residues phosphorylated by A-kinase and CaM-MPK in vitro, and use an improved procedure based on the tumour promoter okadaic acid to identify the protein phosphatases which dephosphorylate TH in vitro and in intact chromaffin cells.

2. EXPERIMENTAL

2.1. *Materials*

TH was purified from bovine adrenal cytosol [10] and the catalytic subunits of protein phosphatases 1 and 2A from rabbit skeletal muscle [11]. The catalytic subunit of A-kinase from bovine heart [12] (Dr C. Smythe), CaM-MPK from rat brain [13] (Dr D.G. Hardie) and inhibitors 1 and 2 from rabbit skeletal muscle [14] (Dr M.J. Hubbard) were provided by other members of the Dundee laboratory. Okadaic acid was obtained from Dr Y. Tsukitani (Fujisawa Chemical Company, Japan).

2.2. *Preparation of tissue extracts*

Bovine skeletal muscle and adrenal glands were obtained from the local abattoir after about 30 min of warm ischemia, and transported to the laboratory on ice. The adrenal cortex and medulla were dissected and these tissues and rabbit corpus striatum homogenized in a Potter-Elvehjem homogenizer in 3 vol. of ice cold buffer containing 50 mM Tris-HCl, pH 7.0, 2 mM EDTA, 2 mM EGTA, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine and 250 mM sucrose. The homogenates were centrifuged for 10 min at $1000 \times g$, and the supernatants removed and centrifuged for 17 min at $15000 \times g$. The supernatant was removed and the pellet resuspended in the same volume of homogenizing buffer. Contamination of adrenal medulla by adrenal cortex and vice versa was $<5\%$. Bovine skeletal muscle was minced and homogenized in a Waring blender for 30 s at low speed using the same buffer except that sucrose was omitted. After centrifugation for 45 min at $4200 \times g$, the supernatant was decanted.

2.3. *Isolation and labelling of chromaffin cells*

Bovine adrenal chromaffin cells were isolated essentially as described [15] and kept in a buffer containing 20 mM Hepes, pH 7.2, 130 mM NaCl, 10 mM KCl, 2.2 mM $CaCl_2$ and 5 mM glucose. Typically, cell viability was greater than 90%, as judged by trypan blue exclusion. The cells (3.4×10^6/ml) were incubated for 90 min at 37°C in [^{32}P]phosphate (0.2 mCi/ml) and 1 μM okadaic acid, then pelleted rapidly and homogenized in 10 vols of ice cold 25 mM EDTA, 25 mM EGTA, 40 mM sodium fluoride, 5 mM sodium pyrophosphate and 0.1 mg/ml leupeptin, pH 7.0. The homogenate was divided into three portions. One was precipitated with 10% (w/v) trichloroacetic acid to measure total ^{32}P-incorporation into protein; the second was used to measure TH activity, while the third was mixed with an equal volume of 1% SDS, incubated for 10 min at 95°C, subjected to SDS-polyacrylamide gel electrophoresis and autoradiographed. TH phosphorylation was then estimated by densitometric scanning of the 60 kDa band on the autoradiograms.

2.4. *Preparation of ^{32}P-labelled protein substrates*

These were prepared with a specific activity of 10^6 dpm/nmol. ^{32}P-labelled bovine adrenal TH was phosphorylated to 0.4–0.6 mol/60 kDa subunit using A-kinase or 0.5 mol phosphate/60 kDa subunit using CaM-MPK [3] and freed from [γ-^{32}P]ATP by gel filtration on Sephadex G50 at ambient temperature as in [16]. Since some preparations of TH contained significant amounts of endogenous protein phosphatase 2A activity, the ^{32}P-labelled enzyme was incubated for 15 min at ambient temperature in the presence of 50 mM NaF and 10 mM sodium pyrophosphate prior to gel filtration. After this treatment, the phosphorylated enzyme could be stored for one week at 5°C with little loss of ^{32}P-radioactivity. ^{32}P-labelled phosphorylase (1 mol phosphate/97 kDa subunit) [11] and ^{32}P-labelled phosphorylase kinase (1.8 mol phosphate/$\alpha\beta\gamma\delta$ unit) [16] (both from rabbit muscle) were prepared by phosphorylation with phosphorylase kinase and A-kinase, respectively.

2.5. *Protein phosphatase assay*

Protein phosphatases were assayed in the absence of divalent cations and presence of 0.1 mM EGTA as in [11,16], although 20 mM magnesium acetate was included to measure type 2C activity where indicated [17]. Caffeine (5 mM) was included when glycogen phosphorylase was the substrate [11]. When inhibitors 1 or 2 were present, diluted enzyme was preincubated for 15 min with inhibitor prior to initiating reactions with substrate [14].

2.6. *Assay of tyrosine hydroxylase activity*

This was measured in potassium-Mes buffers at pH 6.0 and 7.0 [18] using 0.1 mM L-tyrosine as substrate and 1 mM 6-methyl-tetrahydropterin as cofactor.

2.7. *Isolation of phosphopeptides and sequence analysis*

Native ^{32}P-labelled tyrosine hydroxylase was cleaved with trypsin to release trichloroacetic acid-soluble phosphopeptides, which were chromatographed on a Vydac C_{18} column (The Separations Group, Hesperia, CA, USA) equilibrated in 0.1% trifluoroacetic acid as in [3]. The major peptide phosphorylated by CaM-MPK was pure, but that phosphorylated by A-kinase required further chromatography on the Vydac column in ammonium acetate at pH 6.5 before sequence analysis [3]. The peptides were analysed on an Applied Biosystems 470A gas-phase sequencer.

3. RESULTS AND DISCUSSION

3.1. *Identification of phosphorylation sites on bovine adrenal tyrosine hydroxylase*

After phosphorylation by either A-kinase or CaM-MPK, over 90% of the ^{32}P-radioactivity was released from bovine adrenal TH as trichloroacetic acid-soluble peptides by brief tryptic digestion of the native enzyme, as observed previously for rat pheochromocytoma tyrosine hydroxylase. All the ^{32}P-radioactivity was present as phosphoserine, as judged by electrophoresis on thin layer cellulose at pH 1.9 following partial acid hydrolysis (not shown). One major phosphopeptide (peptide A) was obtained after phosphorylation by A-kinase eluting from the C_{18} column at 15% acetonitrile (fig.1), while two phosphopeptides were obtained after phosphorylation by CaM-MPK. The major peptide labelled by CaM-MPK (peptide C) eluted at 13% acetonitrile, while the minor coeluted with peptide A (fig.1).

The sequence of peptide A was $R_{49}Q_{74}S_{53}L_{42}I_{40}$-$Q_{37}D_{43}A_{25}R_{+}$, while that of peptide C was $A_{77}V_{38}S_{13}E_{21}L_{18}D_{19}A_{10}K_{7}$. The values given as subscripts denote pmol of phenylthiohydantoin amino acids detected after each cycle of Edman degradation in the gas-phase sequencer. These sequences correspond to residues 38–46 and 17–24 of the predicted structure of bovine adrenal tyrosine hydroxylase deduced from the cDNA [3,10,19,20], and each differ at one position from the sequences surrounding Ser-40 and Ser-19 of the pheochromocytoma enzyme (the second glutamine in peptide A being replaced by glutamic acid, and the leucine residue in peptide C by glutamine) [3]. The results demonstrate that A-kinase and CaM-MPK phosphorylate the equivalent residues in the bovine adrenal and rat pheochromocytoma tyrosine hydroxylases.

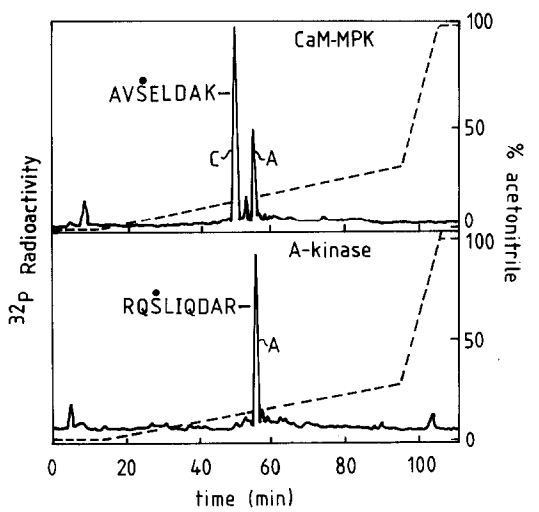

Fig.1. Reverse-phase chromatography of tryptic phosphopeptides (A–C) from bovine adrenal TH. Chromatography was in 0.1% (by vol.) trifluoroacetic acid and developed with an acetonitrile gradient (broken line). The full line shows ^{32}P-radioactivity in arbitrary units determined with an on-line monitor. The upper and lower traces show tryptic peptides obtained from TH phosphorylated by the CaM-MPK and A-kinase, respectively. The radioactivity in the flowthrough fractions is free ATP. The closed circles show the positions of the phosphorylated serines.

Purified bovine adrenal tyrosine hydroxylase contained variable amounts of an endogenous tyrosine hydroxylase kinase activity, which could be inhibited completely by the specific protein inhibitor of A-kinase (not shown), indicating that it is the catalytic subunit of A-kinase.

3.2. *Identification of the major tyrosine hydroxylase phosphatases in extracts of adrenal medulla and corpus striatum*

Four major types of serine/threonine-specific protein phosphatases account for all the phosphatase activity in a variety of mammalian tissues towards a number of phosphoproteins (reviewed in [21]), and improved procedures for identifying and quantitating these enzymes in tissue extracts have been developed recently [22]. Type 1 and type 2A protein phosphatases are active in the absence of divalent cations and can be inhibited specifically by 0.2 μM inhibitor 1 or inhibitor 2 (type 1) and 2 nM okadaic acid (type 2A), respectively. Type 2B (a Ca^{2+}-dependent, calmodulin-stimulated phosphatase) is inhibited by EGTA or trifluoperazine, while type 2C (a Mg^{2+}-dependent phosphatase insensitive to okadaic acid) can be measured specifically in assays containing EGTA and micromolar okadaic acid (which inactivates type 1 as well as type 2A phosphatases).

In the absence of divalent cations and presence of EGTA about 90% of the phosphorylase phosphatase activity in extracts of bovine adrenal medulla or adrenal cortex was catalysed by type 2A phosphatases and only 10% by type 1 phosphatases, as indicated by the percentage of ac-

Table 1

Effect of inhibitor-2, okadaic acid and magnesium ions on phosphorylase phosphatase (PhP) and tyrosine hydroxylase phosphatase (THP) activities in extracts of bovine adrenal medulla, adrenal cortex and skeletal muscle, and rabbit corpus striatum

Additions	Adrenal medulla		Adrenal cortex		Corpus striatum		Skeletal muscle
	PhP activity (%)	THP activity (%)	PhP activity (%)	THP activity (%)	PhP activity (%)	THP activity (%)	PhP activity (%)
1. None	100	100	100	100	100	100	100
2. Okadaic acid (2 nM)	10 ± 2	6 ± 2	10 ± 2	3 ± 2	39 ± 1	6 ± 1	77 ± 3
3. Inhibitor-2 (0.2 μM)	89 ± 2	96 ± 3	86 ± 3	98 ± 2	61 ± 4	97 ± 1	18 ± 1
4. Okadaic acid (2 nM) plus inhibitor-2 (0.2 μM)	3 ± 1	2 ± 1	3 ± 1	2 ± 1	4 ± 1	2 ± 1	2 ± 1
5. Okadaic acid (5 μM)	<1	0	0	0	<1	0	0
6. Okadaic acid (5 μM) plus 20 mM Mg^{2+}	–	28 ± 3	–	24 ± 2	–	17 ± 2	–

Assays were carried out at 1:300 dilution of each extract (1:600 for muscle) using 10 μM ^{32}P-labelled phosphorylase (phosphorylated by phosphorylase kinase) or 1 μM ^{32}P-labelled tyrosine hydroxylase (phosphorylated by A-kinase). Values are given relative to those measured in the standard assay (EGTA without Mg^{2+}) ± standard error for at least three determinations on two preparations. 100% values were: phosphorylase phosphatase, 0.75 ± 0.02 mU/mg (adrenal medulla), 1.4 ± 0.2 mU/mg (adrenal cortex), 5.3 ± 0.9 mU/mg (corpus striatum), 5.5 ± 0.3 mU/mg (skeletal muscle); tyrosine hydroxylase phosphatase, 0.14 ± 0.01 mU/mg (adrenal medulla), 0.22 ± 0.02 mU/mg (adrenal cortex), 0.47 ± 0.03 mU/mg (corpus striatum). One unit of activity is that amount which catalyses the release of 1 μmol of phosphate in 1 min. Very similar results were obtained when inhibitor-2 was replaced by inhibitor-1

tivity inhibited by either 2 nM okadaic acid or 0.2 μM inhibitor 2 (table 1). This type 2A/type 1 ratio of 10:1 with phosphorylase as substrate was about 6-fold higher than in extracts of rabbit corpus striatum and 40-fold higher than in extracts of bovine skeletal muscle where type 1 phosphatases were predominant (table 1). Phosphorylase phosphatase activity was almost completely abolished by the addition of both 2 nM okadaic acid and 0.2 μM inhibitor 2, indicating that type 1 and type 2A phosphatases account for virtually all the activity towards this substrate in each tissue (table 1). The high proportion of type 2A activity in bovine adrenal medulla and cortex was confirmed by the finding that these extracts dephosphorylated the α-subunit of phosphorylase kinase much more rapidly than the β-subunit, and by the finding that nearly all the activity was excluded from heparin-Sepharose [23] (not shown).

When TH (phosphorylated by A-kinase) was used instead of phosphorylase almost all the activity in bovine adrenal medulla and adrenal cortex (table 1), rabbit adrenal gland (not shown) or corpus striatum (table 1) was inhibited by 2 nM okadaic acid, and inhibitor 2 had virtually no effect, demonstrating that type 2A phosphatases account for almost all the TH phosphatase activity under these assay conditions. This was confirmed by the finding that virtually all the TH phosphatase activity in adrenal medullary extracts was excluded from heparin-Sepharose (not shown). Experiments with the purified catalytic subunits of protein phosphatase 1 and protein phosphatase 2A from rabbit skeletal muscle, demonstrated that type 1 had a 40-fold higher phosphorylase phosphatase/TH phosphatase activity ratio than type 2A (not shown), explaining the results in table 1. Homogeneous preparations of the catalytic subunit of protein phosphatase 2A had a similar phosphorylase phosphatase/TH phosphatase activity ratio to that observed in extracts of adrenal medulla (not shown). The weak TH phosphatase activity of the pure catalytic subunit of protein phosphatase 1 could be inhibited completely by 0.2 μM inhibitor 1 or inhibitor 2, demonstrating that it was catalysed by this enzyme and not by traces of contaminating protein phosphatase 2A.

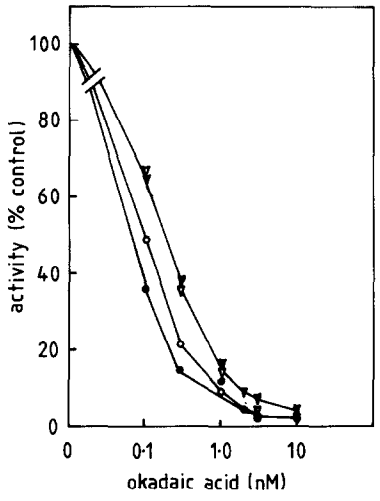

Fig.2. Effect of okadaic acid on the inhibitor-2 insensitive phosphorylase phosphatase and tyrosine hydroxylase phosphatase activities in extracts of bovine adrenal medulla and rabbit corpus striatum. Assays were carried out at a 300-fold final dilution of the extracts in the presence of inhibitor 2. The circles show phosphorylase phosphatase and the triangles tyrosine hydroxylase phosphatase activity. Experiments with adrenal extracts are shown by the open symbols and experiments with striatal extracts by the filled symbols.

The amount of okadaic acid required to inhibit the catalytic subunit of protein phosphatase 2A is similar to the phosphatase concentration in the assays, and the IC_{50} therefore depends on the concentration of this enzyme in the assays [22]. When extracts of adrenal medulla and basal ganglion were diluted 300-fold to give a phosphorylase phosphatase activity of <0.1 mU/ml, the dephosphorylation of this substrate was inhibited with an IC_{50} of about 0.1 nM, as expected [22]. The IC_{50} for okadaic acid with TH as substrate was 0.2 nM (fig.2).

In the presence of 5 μM okadaic acid, the TH phosphatase activity in adrenal medulla, adrenal cortex and corpus striatum was completely dependent on Mg^{2+}, indicating that type 2C protein phosphatases are active towards TH (table 1). However, TH phosphatase activity catalysed by type 2A phosphatases was 3.6-fold (adrenal medulla) and 6-fold (corpus striatum) greater than that catalysed by type 2C enzymes at optimal Mg^{2+} (20 mM). Since the concentration of Mg^{2+} which half maximally activates the TH phosphatase activity of type 2C phosphatases in each extract is 1 mM (not shown), type 2C only accounts for 11% and 7% of the total TH phosphatase activity in dilute extracts of adrenal medulla and corpus striatum, respectively, at near physiological free Mg^{2+} (about 1 mM). Omission of EGTA and inclusion of 0.2 mM $CaCl_2$ did not alter TH phosphatase activity significantly, while trifluoperazine (150 μM) had no effect, indicating that type 2B phosphatases do not contribute significantly to TH phosphatase activity in the extracts (not shown). Inclusion of tetrahydrobiopterin (0.1–3 mM) in the assays had no effect on the TH phosphatase activity of the extracts.

Table 2

Effect of treating intact chromaffin cells with okadaic acid (1 μM) on tyrosine hydroxylase activity and phosphorylation

	TH activity at pH 6.0 (%)	TH activity at pH 7.0 (%)	TH phosphorylation	Protein phosphorylation (total)
Control	100	100	100	100
+ okadaic acid	101 ± 9	177 ± 31	306 ± 17	266 ± 78
+ okadaic acid (in assay only)	99 ± 1	98 ± 9	–	–

TH activities were measured in the cell homogenates and are given as a percentage of those measured in control incubations where okadaic acid was omitted. The values are given ± standard errors for at least six experiments performed on three preparations on chromaffin cells. 100% TH activity values were: 6.4 mU/mg (pH 6.0) and 1.6 mU/mg (pH 7.0). One unit of activity (U) is that amount which catalyses the formation of 1 μmol of DOPA in 1 min. TH activity and phosphorylation, and total protein phosphorylation, were determined as described in section 2

The experiments described above were carried out with TH phosphorylated by A-kinase. However, very similar results were obtained when TH phosphorylated predominantly in peptide C by the CaM-MPK was used. These experiments demonstrate that type 2A phosphatases are the major TH phosphatases in extracts of adrenal medulla and corpus striatum. The structure of the type 2A phosphatase catalytic subunit from bovine adrenal cortex has been deduced from the cDNA [24] and its structure differs at only one position from the rabbit skeletal muscle 2Aα isoform [25].

3.3. *Effect of okadaic acid on the phosphorylation state and activity of tyrosine hydroxylase in bovine chromaffin cells*

Okadaic acid, a C_{38} polyether fatty acid [26], can enter cells, and when added to intact adipocytes or hepatocytes rapidly increases protein phosphorylation and alters carbohydrate and lipid metabolism in the manner expected of a specific protein phosphatase inhibitor [27]. In three different batches of chromaffin cells incubated for 90 min in the presence of 1 μM okadaic acid, total protein phosphorylation was increased 2.7-fold and TH phosphorylation 3.1-fold (table 2). In the same experiments, TH activity (assayed at pH 7) was increased 1.8-fold, while TH activity (measured at pH 6.0) was unaltered. Okadaic acid (1 μM) added directly to the assays had no effect on TH activity (table 2). Unphosphorylated TH has a pH optimum near pH 6.0, while the pH optimum for the phosphorylated enzyme is higher [2]. Thus increased TH activity after treatment of the cells with okadaic acid is likely to be due to increased phosphorylation. Type 2A protein phosphatases have been shown to participate in a variety of metabolic processes [21] and the present work therefore extends its list of functions to include a regulatory role in catecholamine biosynthesis.

3.4. *Conclusions*

The results described in this paper demonstrate that the TH phosphatases present in adrenal medulla and corpus striatum are accounted for by the phosphatase catalytic subunits present in all mammalian cells that have pleiotropic actions [21]. No evidence was found for the presence of specific TH phosphatases. The different peaks of TH phosphatase resolved by anion-exchange chromatography of rat caudate nucleus extracts [9] (a part of the corpus striatum) may represent different type 2A phosphatases in which the catalytic subunit is complexed to other proteins, as observed in other mammalian tissues (reviewed in [21]). Our results demonstrate that type 2A phosphatases account for 89% and 93%, of the TH phosphatase activity in dilute extracts of bovine adrenal medulla and rabbit corpus striatum, respectively, at 1 mM Mg^{2+} and type 2C phosphatases for the remainder. Furthermore, the effects of okadaic acid on TH phosphorylation and activity in isolated bovine chromaffin cells indicate that type 2A phosphatases are the major TH phosphatases in vivo. These observations further emphasize the importance of okadaic acid as a probe for identifying physiologically relevant protein phosphatases, and for identifying cellular processes that are regulated by phosphorylation.

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