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Lack of regulation of aromatic L-amino acid decarboxylase in intact bovine chromaffin cells

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Abstract

Aromatic L-amino acid decarboxylase (AADC) is the second enzyme in the catecholamine biosynthetic pathway, and its activity is generally considered not to be limiting, and therefore not involved, in regulating flux through this pathway. Recent studies showing that its activity can be regulated *in vivo* and that the enzyme can be phosphorylated and activated *in vitro* have raised the possibility that AADC may play more than an obligatory role in catecholamine biosynthesis. In the present study, the phosphorylation and activity of AADC was evaluated relative to that of tyrosine hydroxylase (TH; the first and rate-limiting enzyme in the pathway) in intact bovine chromaffin cells. Treatment of chromaffin cells with elevated potassium, acetylcholine, phorbol dibutyrate, forskolin, or okadaic acid each increased ^{32}P incorporation into TH (after metabolic labeling of ATP pools with $^{32}P_i$) and TH activity. In contrast, as measured in matched samples, ^{32}P incorporation into AADC was not detected and none of the treatments altered AADC activity. Thus, that AADC can be phosphorylated and activated *in vitro* has questionable physiological significance.

Keywords: enzyme activity, metabolic regulation, protein phosphorylation, tyrosine hydroxylase.
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Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in the biosynthesis of catecholamines, and myriad physiological mechanisms exist to regulate its activity (cf. Kumer and Vrana 1996). For example, the phosphorylation of multiple sites by multiple signaling pathways plays an important role therein over the short-term (cf. Salvatore *et al*. 2000, 2001). The second enzyme in this pathway, aromatic L-amino acid decarboxylase (AADC), is also involved in indoleamine and trace amine biosynthesis but is not generally considered to be rate-limiting (cf. Zhu and Juorio 1995; Berry *et al*. 1996), except in the case of trace amines (Dyck *et al*. 1983). Nonetheless, the demonstration in several studies of short-term regulation of AADC activity (Hadjiconstantinu *et al*. 1988; Rossetti *et al*. 1989, 1990; Zhu *et al*. 1992; Cho *et al*. 1997) has rekindled interest in the enzyme and its potential involvement in regulating monoamine biosynthesis. Following from the suggestion that cyclic AMP was involved in modulating AADC activity (Young *et al*. 1993; Duchemin *et al*. 2000) recently demonstrated that mouse brain AADC could be phosphorylated *in vitro* by cAMP-dependent protein kinase (PKA) and that increased activity of recombinant bovine AADC was associated with its PKA-dependent phosphorylation *in vitro*.

While *in vitro* phosphorylation-activity studies can provide important information regarding regulatory mechanisms, results from such studies do not necessarily reflect physiological events. For example, whereas Campbell *et al*. (1986) demonstrated that PKA could phosphorylate TH at Ser153 *in vitro*, this site is not phosphorylated either *in situ* or *in vivo* (Haycock 1990; Haycock and Haycock 1991). Similarly, whereas Ser8 in TH can be phosphorylated *in vitro* by a proline-directed protein kinase (Vulliet *et al*. 1989), its phosphorylation in adult rat striatum *in vivo* is minimal and not regulated by electrical stimulation of the nigrostriatal tract (Haycock and Haycock 1991). Thus, the present studies

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Abbreviations used: AADC, aromatic L-amino acid decarboxylase; HBS, HEPES-buffered saline; PAGE, polyacrylamide gel electrophoresis; PKA, cyclic AMP-dependent protein kinase; SDS, sodium dodecyl sulfate; TPDS-T, Tris-buffered, divalent cation-deficient, Dulbecco's phosphate-buffered saline containing 0.05% Tween 20; TH, tyrosine hydroxylase.

evaluated the effects of physiological and non-physiological secretagogues (acetylcholine, elevated $[K^+]_o$), protein kinase C and PKA activators (phorbol dibutyrate, forskolin), and a protein phosphatase inhibitor (okadaic acid) on TH and AADC activity and phosphorylation.

Materials and methods

Materials

Unless noted otherwise, drugs and chemicals were from Sigma-Aldrich (St Louis, MO, USA), and cell culture reagents and Immunoprecipitin were from Gibco-BRL (Life Technologies, Bethesda, MD, USA). Fresh bovine adrenal glands were from Animal Technologies Inc. (Tyler, TX, USA). Primary antibodies used were affinity-purified polyclonal antibovine AADC (AB1569; Chemicon Intl., Temecula, CA, USA), monoclonal antipeptide AADC (DDC-109; raised against a peptide from exon 1; Sigma-Aldrich), and affinity-purified sheep (Haycock and Waymire 1982) and rabbit (Haycock 1989) anti-TH. AttoPhos AP substrate was from JBL Scientific (San Louis Obispo, CA, USA).

Bovine adrenal medullary chromaffin cells

Chromaffin cells were isolated, purified, and maintained in suspension culture (4×10^6 cells/40 mL/100 mm diameter petri dish) in Ham's F-12 medium supplemented with 10% (v/v) bovine calf serum (Waymire *et al*. 1983). Cells were harvested by centrifugation (300 *g*, 5 min), rinsed by resuspension and recentrifugation in 40 mL HEPES-buffered saline (HBS, pH 7.4 with NaOH; 150 mM NaCl, 15 mM HEPES, 5.5 mM D-glucose, 1.9 mM K_2HPO_4, 1.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.5 mM EGTA, and 0.5 mM ascorbic acid). Cells were reuspended, aliquoted (10^6 cells/30 μL) and pre-equilibrated by incubation for 30 min at 37°C in the absence of treatment. For phosphorylation studies, cells were resuspended in a low-phosphate HBS (3.6 mM KCl, 0.1 mM K_2HPO_4). After pre-equilibration, a 30-μL aliquot of HBS or low-phosphate HBS (containing 0.2 mCi $^{32}P_i$) was added to each sample and preincubation was continued for an additional 90 min at 37°C. Treatments were initiated by adding 60 μL of treatment (at twice final concentration) in buffer to each sample.

Phosphorylation of TH and AADC

Treatments were terminated by adding 40 μL of concentrated stop solution (final concentrations: 1% sodium dodecyl sulfate (SDS), 3 mM EDTA; pH 8.0 with Tris) to each sample and heating in a boiling water bath for 5 min. Prior to immunoprecipitation, an equal volume (100 μL) of solution was added to yield the following composition: 150 mM NaCl, 15 mM NaF, 1.5 mM EDTA, 1 mM EGTA, 2.5% Nonidet P40, 0.5% SDS, 5 mM Tris-HCl (final pH 7.6). Samples were pre-cleared with 10 μL of Immunoprecipitin and incubated for 3 h at room temperature (22–24°C) with 3 μL of immunoprecipitin that had been pre-coated with 0.2 μg affinity-purified rabbit anti-TH or 2.0 μg affinity-purified rabbit antibovine AADC (lot IB/10), conditions determined empirically to provide quantitative immunoprecipitation (see below). TH and AADC immunoprecipitates were collected by centrifugation and rinsed twice by resuspension in Tris-buffered, divalent cation-deficient, Dulbecco's phosphate-buffered saline containing 0.05% Tween 20 (TPDS-T).

Immunoprecipitates were prepared for SDS–polyacrylamide gel electrophoresis (PAGE) by adding 60 μL TPDS-T and 20 μL 4X-concentrated SDS–PAGE sample buffer. Forty-four μL of 4X-concentrated SDS–PAGE sample buffer was added to the immunoprecipitation supernatants. After heating, a 1/10th portion of each sample was subjected to SDS–PAGE and western transfer/blot immunolabeling to determine immunoprecipitation efficiency (see below). The remaining 9/10ths portion was subjected to SDS–PAGE on mini-gels (200 V, 1–1.5 h), after which gels were dried between dialysis membranes, and ^{32}P incorporation was quantitated using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Blot immunolabeling

For quantitation of immunoprecipitation efficiency, SDS–PAGE gels were electrophoretically transferred to nitrocellulose, stained with Ponceau S, quenched/destained for 1 h in TPDS-T containing 1% polyvinylpyrrolidone, and incubated with monoclonal mouse antibovine AADC or affinity-purified sheep anti-TH (diluted in quench solution) for 1 h (Haycock 1993c). After 3 rinses with TPDS-T the nitrocellulose was incubated with either alkaline phosphatase-conjugated, anti-mouse IgG or anti-sheep IgG antibodies. After three rinses with TPDS-T, Attophos AP fluorescent alkaline phosphate substrate was layered over the blots between two plastic transparency sheets, and the reactions were visualized and quantitated using a Storm PhosphorImager. Fluorescence values from experimental values were converted to relative protein levels by interpolation to pooled-tissue standard curves (Haycock 1993a).

TH and AADC activity assays

Samples were rapidly chilled to 4°C and pelleted, and the supernatants were aspirated. For TH activity assays, cell pellets were sonicated (3 × 10 s) in 100 μL ice-cold 0.01% Triton X100 and centrifuged at 30 000 *g* for 20 s (Airfuge; Beckman, Fullerton, CA, USA). Ten microliters portions of the supernatant were assayed for TH activity by measuring the generation of tritiated H_2O from L-3,5-[^{3}H]tyrosine (Amersham Life Sciences, Chicago, IL, USA) as described by Reinhard *et al*. (1986). The reaction was carried out for 5 min at 37°C in 20 mM sodium phosphate (pH 6.8), 5.0 mM ascorbic acid, 0.1 mM [^{3}H]tyrosine (1 μCi/sample), 0.1 mM D,L-6-methyltetrahydropterin, and 1000 U catalase and terminated by the addition of acidified charcoal slurry.

For AADC activity assays, supernatants were prepared as above with the exception that pellets were sonicated in 0.32 M sucrose. Ten microlitre portions of supernatant were incubated at 37°C for 15 min in 20 mM sodium phosphate (pH 7.0), 1 mM mercaptoethanol, 0.2 mM L-DOPA, 0.1 mM EDTA, 0.2 mM ascorbic acid, 0.1 mM pargyline, 10 μM pyridoxal phosphate. The reactions were stopped by adding a 1/10th volume of 10% perchloric acid. After centrifugation, dopamine in the supernatant was quantitated using ESA CouloChem II electrochemical detection of amines separated by RP-HPLC.

Results and discussion

In order to compare ^{32}P incorporation into TH and AADC across treatments, blot immunolabeling techniques were used to establish conditions under which TH and AADC were

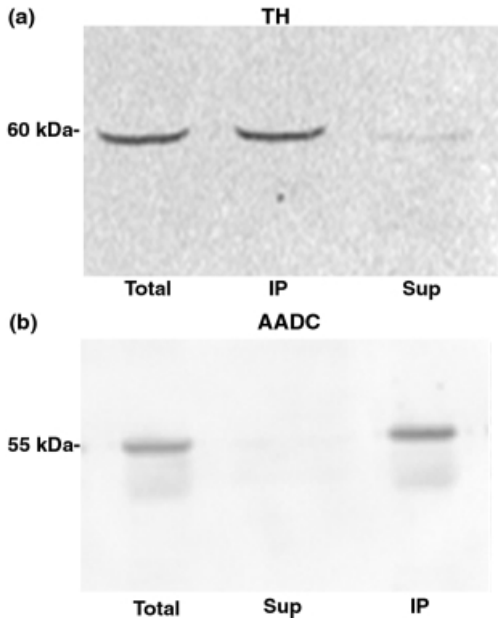

Fig. 1 Quantitative immunoprecipitation of TH and AADC from chromaffin cells. Optimized amounts of Immunoprecipitin-immunobilized affinity-purified rabbit anti-TH and anti-AADC were used to immunoprecipitate (a) TH and (b) AADC from 10^6 bovine adrenal medullary chromaffin cells. Equivalent portions of total cellular protein (Total), immunoprecipitate (IP), and postimmunoprecpitation supernatant (Sup) were subjected to SDS–PAGE and blot immunolabeling as described under Materials and methods. The separation of AADC and IgG heavy chains is illustrated by the heavy chain immunoreactivity present below the 55 kDa AADC band. Quantitation of the blot immunolabeling assay represented in the figure indicated that > 90% of total TH and AADC were both removed from the samples and recovered in the immunoprecipitates (data not shown).

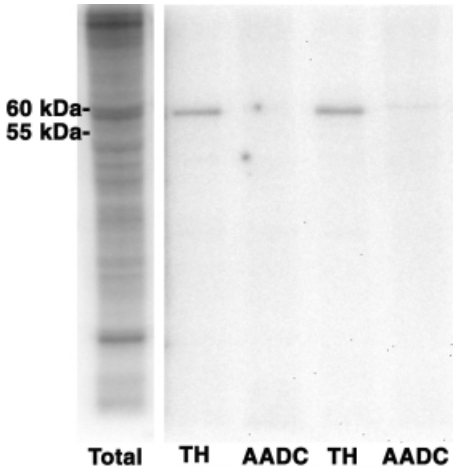

Fig. 2 Basal ^{32}P incorporation into TH and AADC in chromaffin cells. After preincubation for 90 min with $^{32}P_i$, chromaffin cells were either solublized directly with SDS–PAGE sample buffer (Total) or solubilized with SDS and then subjected to immunoprecipitation of TH (lanes 2 and 4) or AADC (lanes 3 and 5) as described under Materials and methods. The lanes shown were loaded with equal chromaffin cell equivalents and the ^{32}P images presented represent identical imaging durations.

quantitatively immunoprecipited from the chromaffin cell extracts (Fig. 1). Figure 2 shows an autoradiogram of ^{32}P incorporation into total cellular proteins and into TH and AADC immunoprecipitated from equivalent amounts of untreated chromaffin cells. Whereas ^{32}P incorporation into TH was comparable to that into the lower of the doublet of ^{32}P-labeled bands in the 60–62 kDa range in the untreated chromaffin cells (cf. Haycock *et al*. 1985), no. ^{32}P-labeled band corresponding to AADC was apparent in the AADC immunoprecipitates – even when exposures were extended long enough to reveal trace amounts of contaminating ^{32}P-labeled bands present in the lane (data not shown; see Fig. 3b, below). A faint band at 61–62 kDa can be seen in lane 5, but not lane 3. This band was observed only occasionally, and unpredictably, and may represent trace amounts of the upper ^{32}P-labeled band of the 60–62 kDa doublet. Notably, it was not immunoreactive in blots with either the sheep anti-TH or the mouse anti-AADC antibodies (data not shown).

The apparent lack of basal ^{32}P incorporation could result from AADC having only a single phosphorylation site with exceedingly low rates of phosphorylation/dephosphorylation. For example, basal stoichiometries of Ser40 can be as low as 0.01 (Salvatore *et al*. 2000, 2001), but TH also has additional phosphorylation sites to contribute to its basal ^{32}P incorporation. However, as shown in Fig. 3, treatment of chromaffin cells with a diverse complement of agents that increase catecholamine biosynthesis increased ^{32}P incorporation into TH (Fig. 3a) but failed to result in detectable ^{32}P incorporation into AADC (Fig. 3b). Note that the ^{32}P autoradiogram of AADC immunoprecipitates in the figure is overexposed to the point of revealing ^{32}P incorporation into trace amounts of a high MW band and what appears to be trace amounts of TH in the AADC immunoprecipitates. Another possible explanation for the failure to detect ^{32}P incorporation into AADC is that the abundance of AADC relative to TH is very low. While this cannot be directly addressed without a sample of known bovine AADC concentration, the activity of AADC in bovine chromaffin cells, both *in situ* (Meligeni *et al*. 1982) and *in vitro* (see below), is orders of magnitude higher than that of TH and an order of magnitude more affinity-purified polyclonal antiprotein antibody was required to quantitatively immunoprecipitate AADC. Lastly, that the affinity-purified anti-AADC failed to recognize PKA-phosphorylated AADC seems unlikely because (1) the antibody would be expected to have multiple epitopes, and (2) Duchemin *et al*. (2000) used the same antibody to immunoprecipitate recombinant bovine AADC prior to phosphorylation with PKA, indicating the presence of epitope(s) distal from the phosphorylation site.

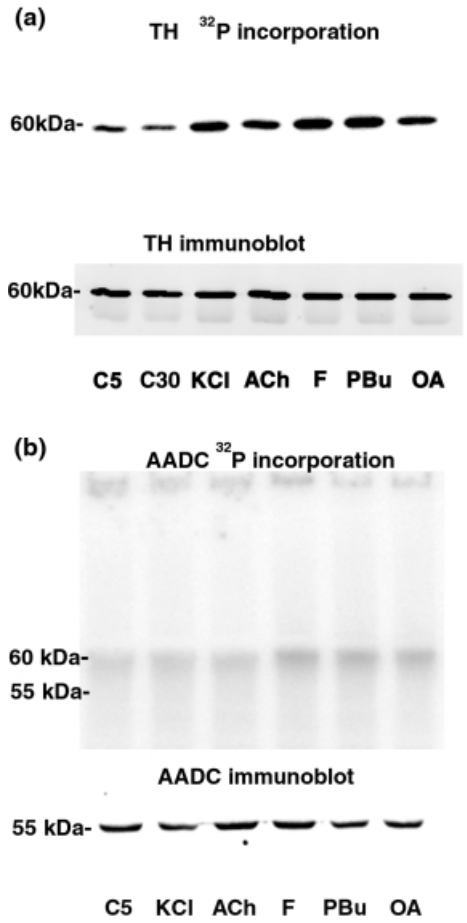

Fig. 3 Treatment-dependent effects on ^{32}P incorporation into TH and AADC in chromaffin cells. After preincubation with $^{32}P_i$ for 90 min, chromaffin cells were treated with 56 mM KCl for 5 min (KCl), 100 μM acetylcholine for 5 min (ACh), 10 μM forskolin for 5 min (F), 10 μM phorbol-12,13-dibutyrate (PBu), or 2 μM okadaic acid for 30 min (OA). Control samples were untreated chromaffin cells taken after 5 min (C5) or 30 min (C30). ^{32}P incorporation and blot immunolabeling (for verifying quantitative immunoprecipitation and validating equivalent loads) of (a) TH and (b) AADC immunoprecipitates were determined as described under Materials and methods.

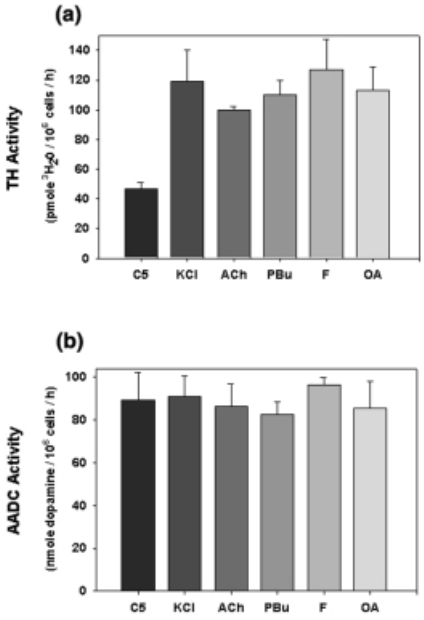

Fig. 4 Treatment-dependent effects on TH and AADC actvity in extracts from chromaffin cells. Chromaffin cells were treated as in Fig. 3 and cell supernatants were assayed for (a) TH and (b) AADC activity *in vitro* as described under Materials and methods. The *x*-axis labels are as in Fig. 3. Values represent the means and error bars represent the standard errors of three determinations.

In that previous studies of AADC regulation have focused on cyclic AMP-related modulation of AADC activity, it should be noted that each of the treatments in Fig. 3 increases Ser40 phosphorylation in TH via, at least to some extent, PKA (Waymire *et al*. 1988; Haycock 1993b), forskolin clearly to the greatest extent. In addition, the relatively prolonged duration of okadaic acid treatment should have provided ample time for even slowly turning over phosphorylation sites to be revealed.

In separate experiments, TH and AADC activity in extracts of chromaffin cells that were treated as in Fig. 3 was measured. While each of the treatments increased TH activity, none was found to have a statistically significant effect on AADC activity (Fig. 4). Although the present AADC activity assays used relatively saturating concentrations of substrate and cofactor, the previously reported increases in AADC activity have been characterized as V_{max} increases (Cho *et al*. 1997) and the PKA-dependent increases in AADC activity reported by Duchemin *et al*. (2000) used similar conditions. Moreover, we have previously demonstrated that in intact intact bovine chromaffin cells, treatment with 8-bromo-cAMP increases the rate of conversion of tyrosine to DOPA but not of DOPA to dopamine (Meligeni *et al*. 1982). Thus, the present lack of regulation of AADC activity *in vitro* is supported by a lack of regulation of AADC activity *in situ* and fails to support previously reported changes in *in vitro* AADC activity from other tissues.

Duchemin *et al*. (2000) clearly demonstrated that AADC, immunoprecipitated from mouse striatum and midbrain, can be phosphorylated *in vitro* by PKA. These authors also demonstrated that PKA can phosphorylate recombinant bovine AADC, in association with a modest (maximally 20%) increase in AADC activity. But, substantial amounts of PKA (30–160 units/50 μL, corresponding to approximately 20–110 μg/mL) were required to produce these effects, and stoichiometries of phosphorylation were not determined, making it difficult to ascertain the extent to which phosphorylation may have been adventitious. However, in any case, the present data clearly indicate that the *in situ* conditions extant in intact chromaffin cells do not mimic those that were necessary to demonstrate the phosphorylation of AADC by PKA *in vitro*.

Regardless of the biochemical considerations above, it was the previous observations that AADC activity could be modulated (see the introduction) that rekindled interest in this enzyme. And, despite the present data – which indicate that AADC activity in chromaffin cells is not modulated by a diverse range of pharmacological treatments – the changes in AADC activity in retina and brain remain unchallenged and of interest. However, the physiological relevance of such changes may be moot. Endogenous DOPA concentrations are normally quite low and the ratio of AADC to TH activity is normally quite high (cf. Figure 4). Cho *et al*. (1997) determined that the activation of AADC was associated with an increase in the V_{max} but not K_m of AADC for DOPA. Considering that the K_ms for DOPA were in the range of 30–40 μM (Cho *et al*. 1997), it is unclear that 20% increases in the V_{max} of AADC would have any effect upon catecholamine biosynthesis *in situ*.

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