Journal of Neuroendocrinology, 2005, Vol. 17, 91–96 doi:10.1111/j.1365-2826.2005.01282.x

Cyclic AMP–Protein Kinase A and Protein Kinase C Mediate *In Vitro* T_3 Activation of Brain Tyrosine Hydroxylase in the Female Catfish *Heteropneustes fossilis*

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Key words: catfish, protein kinase A, protein kinase C, tri-iodothyronine, brain tyrosine hydroxylase.

Abstract

The present *in vitro* study demonstrates an involvement of both cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) signal transduction mechanisms in the triiodothyronone (T_3)-activation of forebrain (telencephalon and hypothalamus) tyrosine hydroxylase (TH) activity in the female catfish *Heteropneustes fossilis*. Incubations of the enzyme preparations with different concentrations of T_3 (0.15–2.4 ng/ml) stimulated TH activity over the concentrations. Similarly, coincubations of the enzyme preparations with T_3 and cAMP (1.0 mM) or cAMP-elevating drugs such as 1-methyl-3-isobutylxanthine (1.5 mM) or theophylline (1.5 mM) increased TH activity significantly over that of T_3. The stimulatory effect of TH activity with T_3 or cAMP was coincident with a low apparent K_m and high V_{max} for the cofactor, suggesting a higher affinity of the enzyme. Incubation of the enzyme preparations with PKA (H-89) and PKC (calphostin-C) inhibitors decreased basal enzyme activity significantly, with the inhibition being greater in the former group. The incubations of the enzyme preparations with T_3 or T_3 + cAMP, followed by the different inhibitors, also decreased enzyme activity. Although T_3 could not reverse the inhibitory effect of H-89, it could over-ride the effect of calphostin-C to some extent. The suppressive effect of the inhibitors could be related to a high apparent K_m and low V_{max} for the cofactor. The evidence strongly suggests a nongenomic action of T_3 on TH activity via the cell signalling pathways, for which the cAMP-dependent PKA appears to be the major regulatory mechanism.

Tyrosine hydroxylase (TH) catalyses the rate-limiting step in the biosynthesis of catecholamines (1). TH activity is influenced by multiple factors such as adrenergic nerve stimulation, stress, end products (e.g. catecholamines), phospholipids, polyanions (e.g. heparin), ferrous ion, catecholestrogens, hormones (e.g. thyroid hormones), oestrogens, calcitonin, glucocorticoids and insulin, growth factors, and drugs such as reserpine (1–4). Enzyme catalytic activity can be modified by altering its functional state by phosphorylation (acute regulation) or by changing its synthesis and/or degradation (long-term regulation) (2, 5). In both regulations, cAMP-dependent protein kinase A (PKA) plays a major and dominant role (2, 6–8). Apart from PKA, TH can be phosphorylated by other kinases such as Ca^{2+}/calmodulin-dependent multiprotein kinase (2, 9), cGMP-dependent protein kinase (10), TH kinase (11) and protein kinase N (12).

Thyroid hormones influence multiple events in neural development, such as axonal maturation, neurite growth, cell migration and myelin formation (13, 14). They have been demonstrated to influence the maturation and maintenance of central catecholaminergic system (15, 16) and sympathetic noradrenergic and adrenal catecholaminergic activity (17). Immunocytochemical studies have demonstrated the coexistence of both thyronergic [triiodothyronone (T_3)-containing] and noradrenergic systems (locus coeruleus neurones and their targets) and T_3 can act as a neurotransmitter/neuromodulator (18, 19). The role of thyroid hormones in morphogenesis, development, growth, osmoregulation, migration, metabolism and reproduction are broadly defined in teleosts (20), but their involvement in any specific functions of the brain is not demonstrated. As in mammals, the teleost brain is also a site for T_4 and T_3 deiodination (21). In a recent study, we showed that brain TH activity and kinetics are influenced by the thyroid status: hypothyroidism inhibits, and hyperthyroidism stimulates, TH activity (22).

Similar to steroid hormones (23), thyroid hormones mediate their effects through nuclear receptors altering the expression of target genes, or act at the plasma membrane level affecting cellular functions within minutes or seconds (nongenomic effects) via second messenger signalling mechanisms (24–27). To the best of our knowledge, a nongenomic action of T_3 on TH activity has not been demonstrated.

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In a previous study, we reported significant seasonal variations in brain TH with peak activity in June (late vitellogenic phase) and sex differences with high activity in females (28). Therefore, the present study was conducted in the gonad recrudescent (vitellogenic) phase in female catfish to demonstrate the involvement of cAMP-dependent PKA and protein kinase C (PKC) on the short-term regulation of TH by T_3 *in vitro*. TH activity and kinetics were correlated to demonstrate a nongenomic action of T_3.

Materials and methods

Chemicals

Catalase, L-tyrosine, 6,7-dimethyl-2-amino−4-hydroxy-5,6,7,8-tetrahydropteridine ($DMPH_4$), bovine serum albumin (BSA), adenosine 3,5′-cyclic monophosphate sodium salt (cAMP), theophylline, 1-methyl-3-isobutylxanthine (IBMX), triiodothyronine (T_3), H-89, calphostin-C and sephadex G-25 were purchased from Sigma (St Louis, MO, USA). Sodium molybdate, 2-mercaptoethanol, sodium nitrite and Folin-Ciocalteu reagent were purchased from E-Merck (Mumbai, India). All other chemicals were of analytical grade.

Collection and acclimatisation

Freshwater air-breathing catfish (*Heteropneustes fossilis*) were collected from local fish markets in Varanasi in gonad recrudescent phase of the annual reproductive cycle. The study was conducted in early (May) and late (June) vitellogenic phase. Female fish (weighing 35–45 g) were selected and acclimated in flow-through aquarium tanks under normal photoperiod and ambient temperature (13 : 11 h light/dark cycle; 28 ± 2 °C) for 15 days. During maintenance, the fish were fed minced goat liver daily.

Experiments

The experiments were performed in accordance with local/national guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind. Acclimated catfish (group size, n = 5) were decapitated between 11 and 12 h for different experiments. Brains, along with pituitary, were dissected out on ice for enzyme preparation. The telencephalon (excluding olfactory tract and bulb) and hypothalamus including pituitary were separated from the brain. Tissues were stored at −70 °C for 24 h. They were thawed and homogenised (50 mg) in 1 ml of 30 mM sucrose containing 10 mM Tris-HCl buffer (pH 7.3) in a Potter-Elvehjem homogeniser with a loose fitting Teflon pestle. The rotor speed was 300–500 r.p.m and the pestle was taken up and down four to five times. The homogenate was centrifuged at 105 000 ***g*** for 1 h and passed through a sephadex G-25 column (1 ml column, flow rate-1 ml per 40 min) at 4 °C to remove endogenous catecholamines. The eluate containing tyrosine hydroxylase was used as the enzyme preparation for incubations described below.

Effects of different concentrations of T_3 on TH activity

T_3 was dissolved in phosphate-buffered saline (PBS, 2.0 M, pH 6.2) to make a stock solution and diluted to different concentrations (0.15, 0.3, 0.6, 1.2 and 2.4 ng/ml). The enzyme preparations were incubated with each concentration of the T_3 in duplicate at 30 °C for 25 min and TH activity was measured. For a control, the incubations were conducted with the respective vehicle medium.

Effects of coincubations of T_3 with cAMP, IBMX or theophylline

The enzyme preparations were coincubated with T_3 (1.2 ng/ml) and cAMP (1.0 mM), IBMX (1.5 mM) or theophylline (1.5 mM) in duplicate at 30 °C for 25 min and TH activity was measured. Control incubations were conducted with the vehicle medium.

Effects of H-89 and calphostin-C and their coincubations with T_3

A concentration–response study of the inhibitors was set up to determine the concentration that gave a maximum effect on TH activity (data not shown). A stock solution of H-89 was prepared in 1 M HCl in PBS buffer (final pH 6.2). The enzyme preparations were incubated at 30 °C with 1, 5 and 10 μM concentrations of H-89 in duplicate for 25 min. A stock solution of calphostin-C was prepared in 2.0 M PBS buffer (pH 6.2). The enzyme preparations were incubated with 1, 5 and 10 μM at 30 °C in duplicate for 25 min. For coincubations, the enzyme preparations were incubated with T_3 (1.2 ng/ml) and 10 μM H-89 or 10 μM calphostin-C in duplicate at 30 °C for 25 min and TH activity was measured. Control incubations were conducted with the vehicle medium or the inhibitors alone.

Effects of coincubations of cAMP + T_3 with the inhibitors

The enzyme preparations were coincubated with cAMP (1.5 mM) and T_3 (1.2 ng/ml) for 10 min, followed by H-89 (10 μM) or calphostin-C (10 μM) for 15 min in duplicate at 30 °C and TH activity was measured. The drugs were added successively in the incubation medium. Control incubations were conducted with the vehicle medium.

Assay of tyrosine hydroxylase

TH activity in the samples of various experiments was measured by the method of Shiman *et al.* (29). The assay was based on the conversion of L-tyrosine to L-DOPA in the presence of the cofactor $DMPH_4$ and molecular oxygen. Absorbance of the colour developed was measured in a spectrophotometer. After the incubations with the test compounds or vehicle, the enzyme preparations were divided into two aliquots (A and B) for the assay of TH activity and kinetics. To the aliquot A, reaction reagents were added in the following order: 0.25 ml L-tyrosine (2 mM), PBS buffer (pH 6.2), 0.01 ml catalase (1 mg per 3 ml in PBS buffer), 0.05 ml of 0.28 M β-mercaptoethanol and 0.05 ml of $DMPH_4$ (6 mM). The reaction mixture was incubated in a test tube at 30 °C for 25 min and 0.5 ml of 0.5 N HCl was added to stop the reaction. Freshly prepared nitrite–molybdate (1 ml) reagent was added to the mixture and allowed to stand for 5 min. The colour was stable for 30 min. Subsequently, 0.5 ml of 2 N NaOH solution was quickly added and mixed. Absorption was immediately determined at 510 nm in a Systronics UV-VIS spectrophotometer. To express enzyme activity, tissue protein content in each sample was measured by the method of Lowry *et al.* (30) using BSA as standard. Enzyme activity was expressed as nmoles of L-DOPA formed/mg protein/h.

Determination of K_m and V_{max}

The aliquot B of the enzyme preparations in the concentration studies and experiments involving T_3, cAMP H-89 and calphostin-C were used for the determination of enzyme kinetics. Michaelis–Menten constants (K_m) and velocity maximum (V_{max}) of the enzyme for the substrate and cofactor were determined from double reciprocal Lineweaver–Burk (LB) plots. The aliquots were incubated in different concentration ranges of L-tyrosine (0.1–0.5 mM) or $DMPH_4$ (1–8 mM) to measure TH activity, as described above. The K_m and V_{max} were calculated from the intercepts ($-1/K_m$ and $1/V_{max}$, respectively) on the 'x' and 'y' axes of the LB plots (Fig. 1).

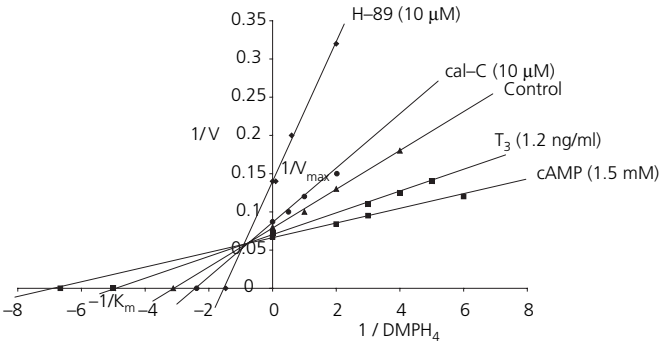

FIG. 1. Lineweaver–Burk plot of hypothalamic tyrosine hydroxylase (TH) for cofactor ($DMPH_4$) in the catfish *Heteropneustes fossilis* showing kinetic responses to T_3, cAMP, H-89 and calphostin-C. 1/V indicates the reciprocal of velocity of the enzyme reaction (measured activity).

Statistical analysis

All data are expressed as mean ± SEM and were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test ($P < 0.05$).

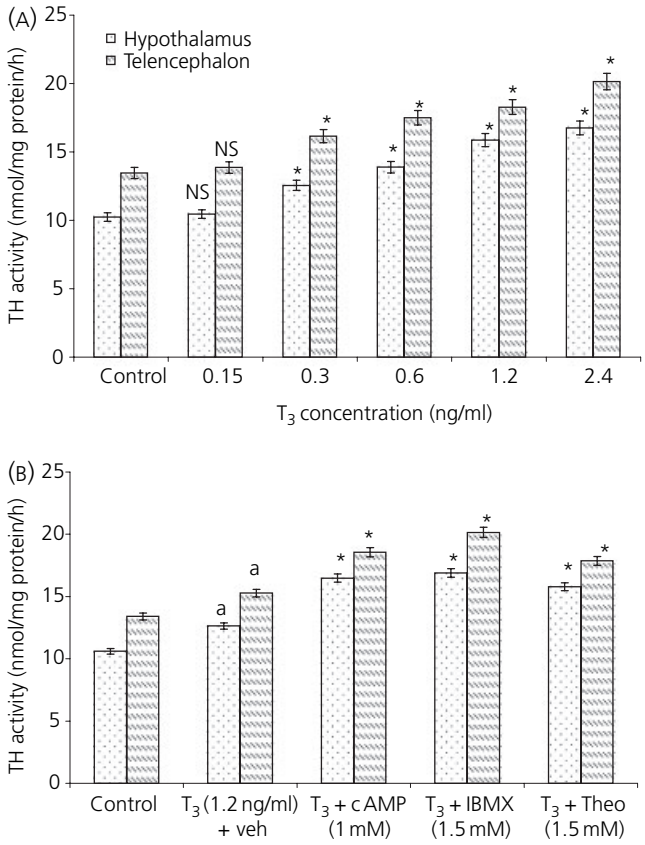

FIG. 2. (A,B) Effects of different concentrations of triiodothyronine (T_3) (A) and coincubations of T_3 (1.2 ng/ml) with cAMP, 1-methyl-3-isobutylxanthine (IBMX) or theophylline (Theo) (B) on hypothalamic and telencephalic tyrosine hydroxylase (TH) activity in the female catfish *Heteropneustes fossilis* (mean ± SEM, n = 5 for each group) in vitellogenic phase (May). Data were analysed by one-way ANOVA ($P < 0.001$) and Tukey's test ($P < 0.05$). Asterisks indicate a significant difference ($P < 0.05$) compared to the control (A) or T_3 + vehicle (B) group. In (B), 'a' above a column indicates a significant difference compared to the vehicle (control) group. NS, Not significant.

Results

Effects of different concentrations of T_3 on TH activity and its kinetics

The incubation of the enzyme preparations with different concentrations of T_3 (0.15–2.4 ng/ml) produced an overall significant effect ($P < 0.001$, one-way ANOVA) on TH activity in the hypothalamus and telencephalon. Enzyme activity increased over the concentrations of T_3 (Fig. 2A) ($P < 0.05$, Tukey's test). Apparent K_m and V_{max} showed overall significant effects for the cofactor only (Fig. 1; Table 1) ($P < 0.001$; one-way ANOVA). The K_m values decreased significantly and V_{max} increased significantly except in the low concentration (0.15 and 0.30 ng/ml) groups ($P < 0.05$, Tukey's test). The kinetic parameters did not elicit any significant effect for the substrate (data not shown).

Effects of coincubations of T_3 with cAMP, IBMX or theophylline

The coincubation of the enzyme preparations with T_3 + cAMP, T_3 + IBMX and T_3 + theophylline produced an overall significant effect on TH activity (Fig. 2B, one-way ANOVA). Enzyme activity increased significantly in all groups compared to the T_3 + vehicle group ($P < 0.05$, Tukey's test).

Effects of PKA and PKC inhibitors and their coincubations with T_3

Incubations of the enzyme preparations with H-89 and calphostin-C inhibited enzyme activity over the concentrations. At 10 μM concentration, H-89 decreased TH activity by 50–55% and calphostin-C by approximately 26%. The coincubations of the enzyme preparations with T_3 + H-89 and T_3 + calphostin-C produced an overall significant effect on TH activity ($P < 0.001$, one-way ANOVA) (Fig. 3A). The enzyme activity decreased significantly in the coincubation groups compared to the vehicle group ($P < 0.05$, Tukey's test). The inhibition was greater in the T_3 + H-89 group. There was no significant difference in telencephalic TH activity between the H-89 and T_3 + H-89 groups. However, T_3 lowered the inhibitory effect of calphostin-C and TH activity was increased over that of the calphostin-C group.

TABLE 1. Effects of Triiodothyronine (T_3) on Apparent K_m and V_{max} of Tyrosine Hydroxylase for Cofactor ($DMPH_4$) in the Hypothalamus and Telencephalon of the Female Catfish *Heteropneustes fossilis* in Vitellogenic Phase (May).

	Hypothalamus		Telencephalon	
Concentrations (ng/ml T_3)	Apparent K_m (mM)	Apparent V_{max} (nmol/mg protein/h)	Apparent K_m (mM)	Apparent V_{max} (nmol/mgprotein/h)
Control	0.36 ± 0.008^1	11.45 ± 1.08^1	0.28 ± 0.002^1	14.62 ± 0.92^1
0.15	0.34 ± 0.002^1	11.82 ± 0.92^1	0.25 ± 0.006^1	14.72 ± 0.82^1
0.30	0.28 ± 0.004^2	12.18 ± 0.62^2	0.20 ± 0.005^2	14.92 ± 0.56^2
0.60	0.22 ± 0.006^3	12.72 ± 0.68^2	0.15 ± 0.004^3	15.82 ± 0.62^3
1.20	0.20 ± 0.002^3	13.88 ± 0.45^3	0.10 ± 0.003^4	16.72 ± 0.82^4
2.40^3	0.18 ± 0.002^3	14.08 ± 0.84^3	0.08 ± 0.004^4	17.81 ± 0.72^5

The K_m and V_{max} were calculated from Lineweaver–Burk plots. Values are means ± SEM. Data were analysed by one-way ANOVA ($P < 0.001$) and Tukey's test ($P < 0.05$). Values with different superscript numbers are significantly different from control and T_3 groups.

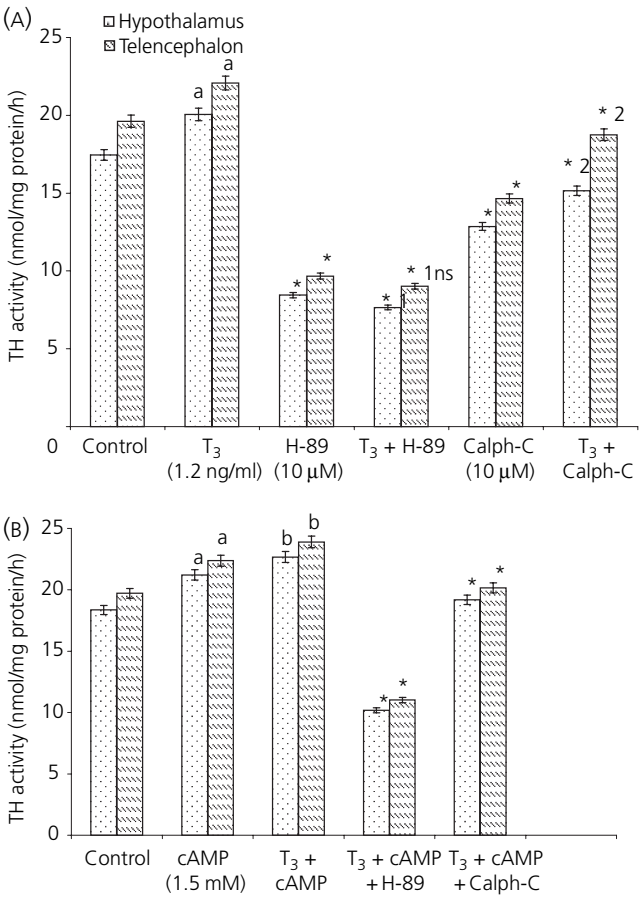

FIG. 3. (A,B) Effects of H-89 and calphostin-C (Calph-C) on hypothalamic and telencephalic tyrosine hydroxylase (TH) activity, alone or in combination with T_3 (A) or T_3 and cAMP (B) in the female catfish *Heteropneustes fossilis* (mean ± SEM, n = 5) in vitellogenic phase (June). Data were analysed by one-way ANOVA ($P < 0.001$) and Tukey's test ($P < 0.05$). Asterisks indicate a significant difference ($P < 0.05$) compared to the T_3 or T_3 + cAMP group. In (A), '1' and '2' above a column indicates comparisons with H-89 and Calph-C groups, respectively. In (B), 'a' above a column indicates a comparison with the control and 'b' a comparison with the cAMP group. NS, Not significant.

Effects of PKA and PKC inhibitors on coincubations with cAMP + T_3

The coincubations of the enzyme preparations with cAMP + T_3, followed by H-89 or calphostin-C produced an overall significant effect on TH activity. The enzyme activity decreased in the inhibitor groups (Fig. 3B) ($P < 0.05$, Tukey's test). The inhibition was higher in the H-89 group.

Effects of cAMP, PKA and PKC inhibitors on enzyme kinetics

Apparent K_m and V_{max} of the enzyme for the cofactor produced an overall significant effect, following incubations with T_3 ($P < 0.001$, one-way ANOVA). Incubations with cAMP decreased the apparent K_m value with a consequent increase in V_{max} (Table 2). The incubation of PKA and PKC inhibitors with T_3 or T_3 + cAMP groups increased the apparent K_m and decreased V_{max} significantly.

Discussion

In our previous study performed *in vivo*, we showed that thyroid hormones influence brain TH activity; with hyperthyroidism increasing and hypothyroidism inhibiting TH activity (22). T_4-induced activation has been attributed to altered enzyme kinetics; the affinity of the enzyme increased significantly for the cofactor. The present study performed *in vitro* also affirms the results obtained *in vivo*, and further examined the mechanism of TH activation during short-term T_3 incubation. The concentration-dependent increase in TH activity with T_3 incubation produced a similar increase in the kinetic properties of the enzyme (low apparent K_m and high apparent V_{max}) for the cofactor. Phosphorylated (active) TH has higher affinity for the cofactor/substrate than the nonphosphorylated enzyme (5, 31). Judging from the pattern of kinetic changes, T_3 might have enhanced the phosphorylation of the enzyme, and hence the elevated activity.

As noted in the Introduction, TH can be phosphorylated by multiple kinases. In the present study, we present evidence

TABLE 2. Effects of Coincubations of Triiodothyronine (T_3; 1.2 ng/ml) or T_3 + cAMP with H-89 or Calphostin C (Calph-C) on Apparent K_m and V_{max} of Tyrosine Hydroxylase for Cofactor ($DMPH_4$) in the Hypothalamus and Telencephalon of the Female Catfish *Heteropneustes fossilis* in Vitellogenic Phase (June).

	Hypothalamus		Telencephalon	
Groups	Apparent K_m (mM)	Apparent V_{max} (nmol/mg protein/h)	Apparent K_m (mM)	Apparent V_{max} (nmol/mg protein/h)
Control	0.32 ± 0.004	12.38 ± 0.65	0.28 ± 0.006	15.68 ± 0.75
T_3	0.20 ± 0.006*	13.86 ± 0.42*	0.15 ± 0.004*	16.94 ± 0.85*
H-89	0.68 ± 0.005*[1]	6.95 ± 0.51*[1]	0.80 ± 0.007*[1]	6.62 ± 0.74*[1]
T_3 + H-89	0.65 ± 0.002*[2]	7.45 ± 0.72*[2]	0.75 ± 0.005*[2]	7.12 ± 0.18*[2]
Calph-C	0.42 ± 0.006*[3]	11.45 ± 0.8*[3]	0.40 ± 0.004*[3]	12.14 ± 0.78*[3]
T_3 + Calph-C	0.52 ± 0.003*[4]	9.84 ± 0.86*[4]	0.58 ± 0.003*[4]	10.60 ± 0.75*[4]
T_3 + cAMP	0.15 ± 0.002*[5a]	15.60 ± 0.98*[5a]	0.14 ± 0.003*[5a]	18.42 ± 0.81*[5a]
T_3 + cAMP + H-89	0.78 ± 0.003*[6b]	6.82 ± 0.65*[6b]	0.85 ± 0.004*[6b]	5.65 ± 0.42*[6 b]
T_3 + cAMP + Calph-C	0.60 ± 0.002*[7c]	8.12 ± 0.35*[7c]	0.58 ± 0.008*[7c]	8.17 ± 0.38*[7c]

Asterisks indicate significant differences compared to control values. Superscript numbers indicate a comparison with the T_3 group. Superscript letters indicate comparisons with the T_3 + cAMP group.

for the involvement of both cAMP-dependent PKA and PKC on T_3 activation of TH. It is well established that cAMP–PKA is the principal phosphorylating mechanism controlling both TH activation (nongenomic) and gene expression (genomic). The incubation of enzyme preparations with cAMP or cAMP elevating drugs stimulated TH activity over that of T_3, indicating a stimulatory effect of cAMP on TH activity. Alterio *et al.* (6) demonstrated that incubation of purified TH with cAMP-dependent PKA enhanced its catalytic activity. PKA inhibitors (H-7, H-8 and H-89) have been shown to inhibit TH activity in other biological systems (3, 32). In our study, H-89 inhibited basal TH activity by 50–55%, indicating the involvement of PKA. The coincubations with T_3 alone or in combination with cAMP did not alter the inhibitory effect of H-89, suggesting that the T_3 stimulatory effect is mediated through the PKA pathway. Thus, T_3 appears to act through the cAMP–PKA pathway to stimulate TH activity. The kinetic data show that the PKA inhibition was due to increased apparent K_m and decreased V_{max}, thereby lowering the affinity of the enzyme for the cofactor. It has also been shown that cAMP–dependent PKA phosphorylates Ser^{40} for all four subunits (2, 6, 7, 33, 34). It is generally accepted that phosphorylation of TH by PKA at Ser^{40} produces a reduction in the K_m value for the cofactor and the resultant high catalytic activity (5). Ligand-mediated regulation of TH, such as that of oestradiol, calcitonin, glucocorticoids and hypoxia, has been attributed primarily to this phosphorylating system (3, 7, 35, 36).

TH is also phosphorylated by PKC at Ser^{40} (5, 33). The involvement of PKC has been demonstrated in the prolactin and neurotensin- induced activation of TH (37), but not that of salmon calcitonin (3). Calphostin-C, a PKC inhibitor, decreased basal TH activity significantly, but to approximately 26%, unlike H-89, suggesting a minor PKC involvement in the enzyme activation. The T_3-induced stimulation of TH activity was inhibited by the PKC inhibitor but to a small scale. Rather, T_3 could over-ride the inhibitory effect of calphostin-C and elevate enzyme activity over that of the inhibitor. The inhibition was due to decreased affinity of the enzyme for the cofactor (increased apparent K_m and decreased V_{max}), suggesting a PKC involvement in the phosphorylation of the enzyme.

A comparison of the effects of H-89 and calphostin-C on TH activity clearly shows that the PKA pathway is the major phosphorylating mechanism in the catfish, as in mammals. Furthermore, T_3 elicits its stimulatory effect through this mechanism. This may explain why T_3 could over-ride the effect of the PKC inhibitor to some extent. The higher inhibition by the PKA inhibitor over that of the PKC inhibitor may be due PKA phosphorylating all four subunits of the enzyme at Ser^{40} (34), whereas PKC phosphorylates only two subunits.

The present study reports the first evidence for a nongenomic action of T_3 in the regulation of TH activity. The T_3 action is mediated preferably through the cAMP–PKA pathway. Because thyroid hormones are involved in the development, differentiation and maturation of the catecholaminergic system, the data presented in this study have wider implications in the T_3 regulation of adrenergic functions.

Acknowledgements

The work was partly supported by financial support from Department of Science and Technology, New Delhi to K.P.J.

Accepted 26 January 2005

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