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Application of HPLC with Electrochemical Detection to Assaying Tyrosine Hydroxylase Activity and Dopamine Content in Dissociated Cultures of Fetal Rat Brainstem

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ABSTRACT

We measured the developmental increase of tyrosine hydroxylase(TH) activity and dopamine content with high performance liquid chromatography with electrochemical detection(HPLC-EC) in dissociated cultures of fetal rat brainstem(E14). TH activity and dopamine content increased progressively upto 7 days in vitro, when the effects of various drugs on the dopamine contents were studied. α-Methyl-p-tyrosine, a TH inhibitor and NSD-1015, an inhibitor of aromatic amiono acid decarboxylase effectively depleted dopamine contents. Dopamine contents were depleted by reserpine and increased by pargyline. When cultures grown for 1 week in control medium were then exposed to tetrodotoxin(0.1 μM) for 7 days, exposure to tetrodotoxin markedly decreased TH activity. All the above results indicate that dopamine metabolism in the cultered cells reflect reliably the property of brain dopamine metabolism. We suggest that measuring TH activity and dopamine content in brainstem culture with HPLC-EC can be useful tool in the study of pharmacology as well as toxicology of the central dopaminergic system.

Key Words: Dopamine, Tyrosine hydroxylase, High performance liquid chromatography-electrochemical detection, Dissociated culture of fetal rat brainstem

INTRODUCTION

Tyrosine hydroxylase(TH) activity and dopamine content are phenotypic markers for dopaminergic neurons. Measuring these two markers in cultured cells has been largely dependent on radioenzymatic method, due to the limited amount of cells for assay, especially in dissociated monolayer culture(Sumners *et al.*, 1983). High performance liquid chromatography with electrochemical detection(HPLC-EC) technique has about equal sensitivity to radioenzymatic method in assaying catecholamines(Keller *et al.*, 1976), but has not been utilized as popularly in catecholaminergic neuronal cell cultures as the radioenzymatic method.

In the present study, we applied HPLC-EC technique to measuring TH activity and dopamine content in dissociated cells of fetal rat brainstem. And we could monitor the developmental increase in TH activity and dopamine levels in culture and confirmed the effects of various drugs on dopamine contents as well as the effect of longterm exposure to tetrodotoxin on TH activity.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium/F12

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(DMEM/F12), poly-D-lysine HBr(mol. wt.〉 300, 000), cytosine arabinofuranoside, dopamine, α-methyl-p-tyrosine, 3,4-dihydroxybenzylamine (DHBA), pargyline and tetrodotoxin were ordered from Sigma Chemical Co.(St. Louis, MO, USA); NSD-1015(3-hydroxybenzylhydrazine HCl) from Aldrich Chemical Co.(Milwaukee, Ml, USA); fetal bovine serum from Gibco(Grand Island, NY, USA); reserpine from Aju Pharmaceut. Co.(Seoul) and all other reagents were of analytical grade.

Cell cultures

The procedures for obtaining timed-mated pregnant rats(Sprague-Dawley) and for dissociated cultures of fetal rat brainstem have been described in detail in Kim *et al.*(1989, 1990). Briefly, 3 or 4 rats were sacrificed at the gestational day 14.5-15.5, and brainstems were dissected from 30-50 embryos aseptically under the dissecting microscope.

After careful removal of the meninges, pooled brainstems were rinsed with DMEM/F12 and transfered to the nylon mesh bag. Dissociated cells were obtained by gently pressing the bag with a glass rod in 10 ml of DMEM/F12. After 2 additional washes with 10 ml of DMEM/F12 by spinning at 500 g for 3 min, the final pellet was resuspended in DMEM/F12 supplimented with 10% fetal bovine serum. The cells were plated at an approximate density of 10^5 cells/mm^2 onto 35 mm dishes(Corning) previously coated with polylysine(25 μg/ml). The cells were maintained at 37°C, in a water saturated 5% CO_2 95% air atmosphere. The cultures were treated with 10 μM cytosine arbinofuranoside for 24 h on the 3rd day to suppress growth of glial cells. Thereafter medium was changed every 3 days.

Assay of dopamine

Dopamine was separated and quantitated by HPLC-EC according to the method of Keller *et al.*(1976). A Waters electrochemical detector (Model 460) with glassy carbon electrode(Waters Assoc., Milford, MA, USA) was used. In brief, the cells in each were harvested with cell scraper into 100-200 μl of 0.1M perchloric acid (4°C, containg 0.1% sodium metabisulfite and 50 ng DHBA as internal standard). Cells were homogenized by sonicating for 10 seconds(2 times) in 1.5 ml Eppendorf tubes. Aliquot of 5-10 μl were used for protein assay(Lowry *et al.*, 1951). After adding activated alumina(100-200 mg) and 1 ml of 0.5 M Tris(pH 8.6), the Eppendorf tubes containing homogenate(100-200 μl) were shaken for 15 min. After washing the alumina 3 times with 1 ml of ice-cold distilled water containg 0.1% sodium metabisulfite, dopamine was eluted from alumina by shaking for 15 min with ice-cold perchloric acid(0.1% sodium metabisulfite). After centrifuging at 15,000 g for 20 min, the supernatant(10 μl) was injected onto a 10 1 μm, 30 cm×4.6 mm C_{18}μ BondaPak column using U6K injector(Waters Assoc.). As a mobile phase 10 mM phosphate buffer/5% acetonitrile(0.1 mM EDTA: 0.5 mM sodium octanesulfonic acid) was used at a flow rate of 1 ml/min and the oxidation potential was 0.65 V. Dopamine values were caculated from the ratio of height of dopamine peak relative to that of internal standard(DHBA).

Assay of tyrosine hydroxylase activity

Assay of TH activity was performed according to the procedure of Nagatsu *et al.*(1979). The cells in each dish were harvested with cell scraper into 100-200 μl of 5 mM Tris buffer(pH 7.6, 4°C) containg 0.2% Triton X-100. Cells were homogenized by sonicating for 10 seconds (2 times) in 1.5 ml Eppendorf tubes. Aliquot of 5-10 μl were used for protein assay(Lowry *et al.*, 1951). The standard incubation mixture consisted of the following components in a volume of 100 μl in final concetrations: 50 mM potassium phosphate buffer, pH 6.0, 1 mM 6-methyl-5,6,7, 8-tetrahydropterin in 100 mM 2-mercaptoethanol, 1 mM ferrous ammonium sulfate, 100 μM L-tyrosine and 30-50 μl of homogenate as the enzyme. For blank incubation, D-tyrosine was used as substrate(100 μM) instead of L-tyrosine and 100 pM DOPA were added to another blank incubation as an internal standard for DOPA. The TH inhibitor 3-iodo-L-tyrosine(100 μM) was added to both blanks in order to prevent DOPA-formation from D-tyrosine which contains some L-tyrosine. Incubation was done at 37°C for 20 min. and the reaction was stopped with perchloric acid(4°C) containing 100 pmol α-mehtyldopa as an internal standard.

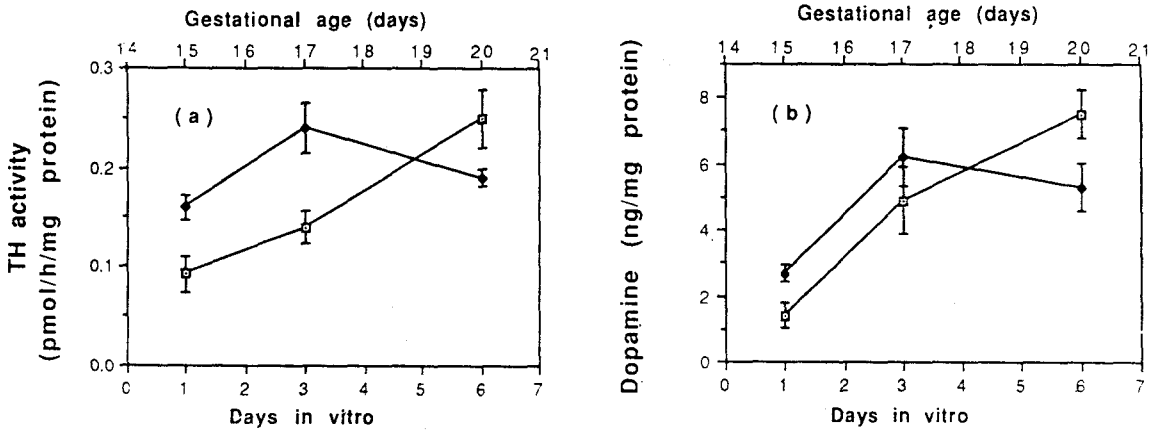

Fig. 1. Developmental patterns of tyrosine hydroxylase activities(a), dopamine contents(b) in primary cultured brainstem neurons and in embryonic brainstems. Each value represents mean ± SEM obtained form 6-8 separate experiments.

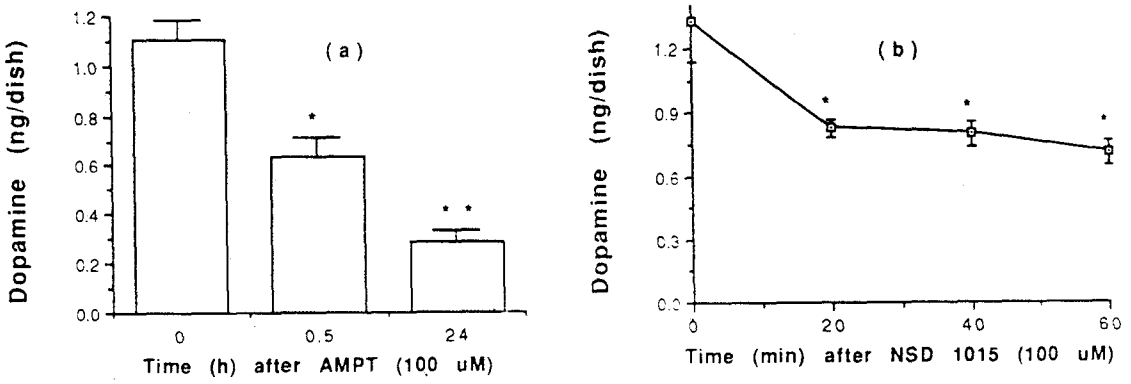

Fig. 2. Effect of(a) α-methyl-p-tyrosine(100 μM) and(b) 3-hydroxybenzylhydrazine(100 μM) on dopamine contents in primary cultured neurons on 7-8th day in vitro. Each value represents mean ± SEM obtained from 3-9 separate experiments. *P<0.05, **P<0.01 compared with control values.

RESULTS

Developmental patterns of TH and dopamine contents

TH activity and dopamine content increased progressively upto 6 days *in vitro*. The TH activity and dopamine contcentration in culture were about in the same range with those of embryonic brainstem corresponding to the same age (Fig. 1).

Effects of several drugs that affect catecholamine metabolism on dopamine contents

After incubation of 6-day-old cultures with various drugs for various time period, dopamine contents were measured.

Incubations of cultures with 100 μM α-methyl-p-tyrosine(AMPT), a specific TH inhibitor, for 0.5 and 24 h, time-dependently decreased dopamine contents(Fig. 2a). Incubations of cultures with 100 μM NSD-1015, a specific AADC inhibitor, for 0-60 min, also time-dependently de-

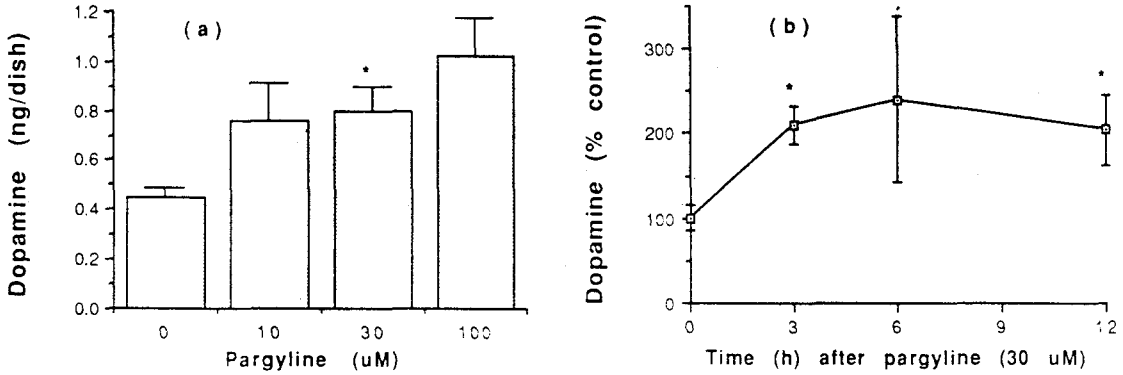

Fig. 3. Dose-dependent increase of dopamine content after 3 hr-incubation with pargyline on 7th day in vitro. Each value represents mean ± SEM obtained from 3-4 seperate experiments. *P<0.05, **P<0.01 compared with control values.

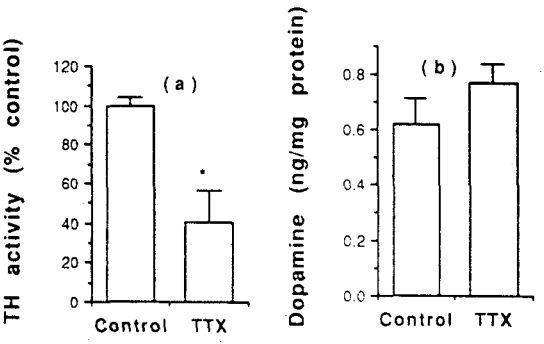

Fig. 4. Effect of tetrodotoxin(TTX, 0.1 μM) treatment on tyrosine hydroxylase activities(a) and dopamine contents(b). Cultures were grown for 1 week and were then treated with TTX for 7 days. Each value represents mean ± SEM obtained from 7 experiments.

creased dopamine contents(Fig. 2b). Incubation of cultures with 10 μM reserpine, a depletor of monoamine for 48 h decreased dopamine contents to 5% of control levels. Incubation of cultures with 0-100 μM parglyine, a MAO inihibitor, for 3 h, increased dopamine contents dose-dependently(Fig. 3a). The amount of increase in dopamine contents by 30 μM parglyine was about the same from 3 h upto 12 h, implying negative feedback regulation of dopamine synthesis(Fig. 3b).

Effects of tetrodotoxin on TH activity

Cultures were grown for 1 week in control medium, and were then exposed to tetrodotoxin (0.1 μM) for 7 days. Exposure to tetrodotoxin markedly decreased TH activity($P<0.05$). On the other hand, dopamine contents tended to increase, but the increase did not reach statistical significant level(Fig 4).

DISCUSSION

Dopamine in the brain is a marker of dopaminergic neurons but norepinephrinergic neurons can also contain dopamine as an intermediate metabolite. Dopamine measured in this study most probably originated from both sourses.

In the culture system, measuring TH activity or dopamine content was frequently relyed on radioenzymatic methods due to the limited amout of tissue for assay, especially in dissociated monolayer culture(Sumners *et al.*, 1983; Pettmann *et al.*, 1979; Prochiantz *et al.*, 1979, 1981). The above-mentioned reports were conducted with cultures the celldensities of which were 100,000-200,000 cells/cm^2. In our cultures, the cell density was in the range of 300,000-400,000 cells/cm^2. And we could detect without difficulty both indexes per 35 mm-dish with

HPLC-EC. But solvent front in our HPLC system interfered norepinephrine peak, so we could not measure norepinephrine levels routinely except in a few samples.

The rapid changes in dopamine contents by drugs(Fig. 2 & 3) suggest that cultured cells are active in dopamine metabolism. In this regard, the dopamine level in the 18-day-old fetal brain has been shown to be as reponsive as the adult rat brains to the effects of α-mehtyl-p-tyrosine, reserpine, and pheniprazine(Coyle and Henry, 1973).

Rather unexpectedly, NSD-1015 was as potent as α-methyldopa in decreasing dopamine contents. This finding suggests that aromatic amino acid decarboxylase may function as rate-limiting step transiently at this develpomental stage in culture due to the incomplete ontogeny of the enzyme. The study of ontogeny of AADC in this culture system may clarify this point.

All the above results indicate that dopamine metabolism in the cultured cells reflect reliably the property of brain dopamine metabolism. But to clarify the origin of dopamine i.e. from either dopaminergic neurons or noradrenergic neurons, it would be necessary to culture more specified brain regions, such as midbrain for dopaminergic neurons and pons for noradrenergic neurons.

Although the mechanism(s) of inhibitory action of tetrodotoxin on TH activity was not investigated in this study, this finding suggests that the basal levels of depolarization or Na^+ influx, which was blocked by tetrodotoxin, mediate the ontogenic rise in TH activity in culture, as reported in explant culture of locus coeruleus by Dreyfus *et al.*(1986).

We suggest that measuring TH activity and dopamine content in brainstem culture with HPLC-EC can be useful tool in the study of pharmacology as well as toxicology of the central dopaminergic system.

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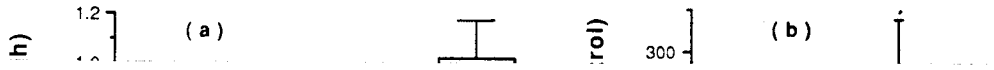

= 국문초록 =

흰쥐 태 뇌간의 세포배양에서 HPLC-전기화학검출을 이용한 Tyrosine Hydroxylase 활성 및 Dopamine의 정량

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쥐(태령 14일) 뇌간 세포배양에서 발달과정에 따른 Tyrosine hydroxylase(TH) 활성 및 dopamine의 양적 증가를 전기화학 HPLC를 이용하여 측정하였다. TH 활성 및 dopamine의 양은 배양 7일까지 점차 증가하였다. 배양 7일째에 여러 약물들의 dopamine 대사에 대한 영향을 조사하였다. TH 억제제인 α-methyl-p-tyrosine 및 aromatic amiono acid decarboxylase 억제제인 NSD-1015는 효과적으로 dopamine을 고갈시켰다. Dopamine은 reserpine에 의해 고갈되었고, parglyine에 의해 증가되었다. 일주일 배양한 세포의 배양액에 tetrodotoxin(0.1 μM)을 7일간 투여하였을 때 TH 활성은 현저히 감소하였다. 이상의 결과들은 배양세포의 dopamine 대사가 뇌 dopamine 대사를 충실히 반영함을 나타낸다. 뇌간 세포의 배양에서 HPLC-전기화학검출을 이용한 TH 활성 및 dopamine의 측정은 중추 dopamine계의 약리 및 독성 연구에 유용하리라 사료된다.