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Does cyclic AMP-dependent phosphorylation account for the activation of tyrosine hydroxylase produced by depolarization of central dopaminergic neurons?

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Tyrosine hydroxylase (EC 1.14.16.2; tyrosine-3-mono-oxygenase) is believed to be the rate-limiting enzyme in the biosynthesis of catecholamines in the central nervous system [1]. As such, it has been the subject of a number of investigations directed at determining the role this critical enzyme plays in maintaining adequate levels of transmitter during periods of increased neuronal activity. Early studies showed that periods of increased impulse flow resulted in an accelerated synthesis of catecholamines and this was believed to arise primarily as a result of the removal of tyrosine hydroxylase from end-product inhibition subsequent to the release of transmitter [2,3]. However, recent studies have shown that brief periods of electrical stimulation of noradrenergic or dopaminergic neurons in the brain lead to a kinetic activation of tyrosine hydroxylase isolated from brain areas in which these neurons terminate [4,5]. This kinetic activation seems to be mediated by a decrease in the apparent K_m of the enzyme for both substrate tyrosine and pteridine cofactor and by an increase in the K_i for inhibitors with a catechol moiety. Moreover, chemical or electrical depolarization of slices prepared from rat striatum and hippocampus also resulted in kinetic alterations in tyrosine hydroxylase similar to those observed upon electrical stimulation of dopaminergic and noradrenergic pathways in the brain [6,7]. These observations raised the question as to the mechanism by which neuronal depolarization activates tyrosine hydroxylase.

Recent evidence indicates that the exposure of either brain slices or synaptosomes to dBcAMP* or the incubation of crude soluble preparations of enzyme from rat striatum or hippocampus under phosphorylating conditions (Mg^{2+}, ATP and cAMP) results in kinetic changes in tyrosine hydroxylase similar to those observed following neuronal depolarization [8–13]. Since chemical or electrical depolarization of brain cortical slices produces an accumulation of cAMP in the tissue, the hypothesis was put forward that the activation of tyrosine hydroxylase by nerve stimulation might be mediated primarily by a mechanism involving a cyclic nucleotide-dependent phosphorylation process [14]. However, the accumulation of cAMP in brain slices following neuronal depolarization most likely occurs in the post-synaptic cells [15], whereas tyrosine hydroxylase is located mainly presynaptically. In an attempt to determine if cAMP-dependent phosphorylation is involved in the activation of tyrosine hydroxylase produced during depolarization, we have assayed, under protein-phosphorylating conditions, crude tissue preparations of tyrosine hydroxylase obtained from striatal slices previously subjected to optimal K^+-depolarizing concentrations. As a comparison, we have also studied the effects of incubating crude enzyme preparations obtained from slices exposed to optimal concentrations of dBcAMP, under phosphorylating conditions. The results are not in keeping with the suggestion that cAMP-dependent phosphorylation plays a primary role in the activation of tyrosine hydroxylase induced by neuronal depolarization.

Preparation and incubation of rat striatal slices. Striata were rapidly dissected from the brain of adult male Sprague–Dawley rats. Tissue slices (300–400 mg; 0.2 mm in thickness) were prepared with a Sorvall tissue chopper and incubated for 15 min at 37°C in 5.0 ml of prewarmed incubation medium saturated with 95% O_2/5% CO_2. At the end of the incubation period the slices were separated from the medium by passage under vacuum through membrane filters which contained a nylon mesh top (pore size, 35 μm). The nylon mesh containing the slices was immediately frozen on dry ice and stored at −70° until the time of tyrosine hydroxylase assay. The KRP media used had the following composition: NaCl, 1.28 mM; KCl, 4.8 mM; $CaCl_2$, 1.3 mM; $MgSO_4$, 1.2 mM; Na_2HPO_4, 15.8 mM at pH 7.4; and dextrose, 11.1 mM. Potassium-enriched KRP was prepared by replacing a portion of the NaCl with an equimolar amount of KCl. Dibutyryl cyclic AMP-enriched KRP was prepared by adding the cyclic nucleotide directly to the KRP media.

Preparation and assay of tyrosine hydroxylase. Frozen striatal slices were homogenized in 20 vol. of ice-cold 0.01 M MES buffer, pH 6.0, containing Triton X-100 (0.2%, w/v). The homogenates were centrifuged at 20,000 *g* for 20 min at 4°, and the supernatant fraction served as the source of soluble tyrosine hydroxylase. Enzyme activity was assayed according to the method of Shiman *et al.* [16] with minor modifications [17]. The reaction was carried out in a total volume of 0.3 ml; 0.05 ml of the supernatant fraction was added to a reaction mixture containing 15 μmoles of MOPS buffer, pH 7.0; 3700 units of catalase; 0.01 ml (2.2 mg protein) of partially purified sheep liver dihydropteridine reductase [18]; 0.15 μmole of $NADPH_2$ and 0.075 μmole of 6-MPH_4. After a 5-min preincubation at 37°, the reaction was initiated by the addition of 3.0 μCi of L-[3,5-^{3}H]tyrosine (1 Ci/mmole) previously purified according to the method of Coyle [19] and evaporated to dryness just prior to use. After a 30-min incubation at 37°, the reaction was stopped by the addition of 0.2 ml of 10% TCA (w/v). Blank values were determined by running the assay in the absence of enzyme. Analysis of the tritiated water formed during the reaction was carried out by ion exchange chromatography on a Dowex 50 × 8 [H^+] column [20]. Tyrosine hydroxylase activity is expressed as pmoles of DOPA formed/min/mg of protein. When the reaction mixture contained $MgCl_2$, ATP and cAMP, the amounts added were 3 μmoles, 0.3 μmole and 15 nmoles respectively. Protein was determined by the method of Lowry *et al.* [21]. Data were analyzed, unless stated differently, by a two-tailed *t*-test using $P < 0.05$ as a level of significance.

Incubation of striatal slices in high potassium KRP resulted in a marked increase in the activity of tyrosine hydroxylase found in the high speed supernatant fraction of homogenates prepared from the slices (Table 1). Potassium-depolarizing concentrations of 20 mM produced an increase in enzyme activity of about 280 per cent when compared to the activity of tyrosine hydroxylase found in striatal slices incubated in normal KRP media. Increasing the external K^+

* Abbreviations used: dBcAMP, dibutyryl cylic AMP; cAMP, cyclic AMP; KRP, Krebs–Ringer phosphate; MES, morpholinoethane sulfonic acid; MOPS, morpholinopropane sulfonic acid; 6-MPH_4, DL-6-methyl-5,6,7,8-tetrahydropteridine HCl; TCA, trichloroacetic acid; and DOPA dihydroxyphenylalanine.

Table 1. Activity of tyrosine hydroxylase isolated from striatal slices previously subjected to potassium depolarization or incubated in dibutyryl cyclic AMP-enriched media

Incubation conditions of the slices *	Tyrosine hydroxylase activity† (pmoles DOPA/min/mg protein)	Tyrosine hydroxylase activity (% control)
KRP	5.7 ± 0.3 (6)	100 ± 5.7 (6)
KRP + K^+ (20 mM)	15.9 ± 0.3 (4)	281 ± 4.8 (4)
KRP + K^+ (40 mM)	15.9 ± 2.5 (4)	281 ± 44.4 (4)
KRP + K^+ (60 mM)	17.4 ± 2.5 (4)	307 ± 44.6 (4)
KRP + K^+ (100 mM)	19.3 ± 0.7‡ (4)	342 ± 12.2‡ (4)
KRP	8.0 ± 0.9 (6)	100 ± 11.5 (6)
KRP + dBcAMP (0.1 mM)	7.9 ± 0.7 (4)	98 ± 8.5 (4)
KRP + dBcAMP (0.5 mM)	11.8 ± 1.6 (4)	147 ± 19.8 (4)
KRP + dBcAMP (1 mM)	15.9 ± 2.8 (4)	199 ± 34.4 (4)
KRP + dBcAMP (3 mM)	21.6 ± 1.8 (4)	270 ± 22.8 (4)
KRP + dBcAMP (5 mM)	28.8 ± 2.0 (4)	359 ± 26 (4)
KRP + dBcAMP (10 mM)	28.6 ± 2.3§ (4)	357 ± 29§ (4)

* Striatal slices were prepared by means of a Sorvall tissue chopper and incubated for 15 min at 37° in the various media. Thereafter, the slices were homogenized in ice-cold 0.01 M MES buffer, pH 6.0, containing Triton X-100 (0.2%, w/v) and assayed for tyrosine hydroxylase.

† Tyrosine hydroxylase was determined in the 20,000 *g* supernatant fraction of the homogenate. Assays were conducted at pH 7.0 in the presence of 10 μM tyrosine and 250 μM 6-MPH_4. Results are expressed as means ± S.E.M. The number of experiments (each one assayed in triplicate) is indicated in parentheses.

‡ Not significantly different when compared to KRP-K^+ (60 mM).

§ Not significantly different when compared to dBcAMP at a 3 or 5 mM concentration.

concentration (up to 100 mM) produced a slightly greater augmentation in the activity of the enzyme. Maximal activity of the enzyme was reached in the presence of an external potassium concentration of 60 mM. Tyrosine hydroxylase activity obtained under this latter condition did not significantly differ from the enzyme activity observed after incubating striatal slices in KRP containing 100 mM potassium. The addition of dBcAMP to the KRP media resulted in a concentration-dependent increase in tyrosine hydroxylase activity assayed in the homogenates prepared from the slices (Table 1). Maximal tyrosine hydroxylase activities were obtained when incubating the slices in the presence of 3–5 mM dBcAMP (about a 270–350 percent increase). In fact, the addition of 10 mM dBcAMP to the KRP produced no further significant increase in the activity of tyrosine hydroxylase when compared to 3 or 5 mM dBcAMP.

Table 2 shows the effects on tyrosine hydroxylase of exposure of the homogenates, obtained from striatal slices previously incubated under normal KRP, optimal K^+-depolarizing concentrations, or optimal dBcAMP concentrations, to phosphorylating conditions. Exposure to phosphorylating conditions (Mg^{2+}, ATP and cAMP) of the tyrosine hydroxylase extracts prepared from slices incubated in normal KRP resulted in a highly significant increase in the activity of the enzyme (Table 2). If cAMP-mediated phosphorylation plays a primary role in the K^+-depolarization-induced activation of

Table 2. Effects of cAMP-dependent phosphorylation conditions on the activity of tyrosine hydroxylase isolated from striatal slices previously incubated under optimal concentrations of potassium and dibutyryl cAMP

Incubation conditions of the slices *	Tyrosine hydroxylase activity† (pmoles DOPA/min/mg protein)		Absolute increase in tyrosine hydroxylase activity due to Mg^{2+}, ATP, cAMP‡ (pmoles DOPA/min/mg protein)
	Standard assay	Standard plus Mg^{2+}, ATP, cAMP	
KRP	9.0 ± 0.4	22.1 ± 0.9§	13.1 ± 0.5
KRP + K^+ (100 mM)	15.8 ± 1.5	32.9 ± 5.1‖	17.1 ± 3.7
KRP + dBcAMP (10 mM)	23.1 ± 0.9	43.0 ± 0.9	19.9 ± 2.9

* Striatal slices were prepared by means of a Sorvall tissue chopper and incubated for 15 min at 37° in the various media. Thereafter, the slices were homogenized in ice-cold 0.01 M MES buffer, pH 6.0, containing Triton X-100 (0.2%, w/v) and assayed for tyrosine hydroxylase.

† Tyrosine hydroxylase was determined in the 20,000 *g* supernatant fraction of the homogenate. Assays were conducted in the presence of 10 μM tyrosine and 250 μM 6-MPH_4, respectively, and in the absence (standard assay) and presence of Mg^{2+} (10 mM), ATP (1 mM) and cAMP (0.05 mM). Results represent the means ± S.E.M. of four different experiments each assayed in triplicate.

‡ One-way analysis of variance for group of slices incubated under the three different incubation conditions: $F = 1.54$, degrees of freedom = 2/9, and $P > 0.05$.

§ $P < 0.001$, when compared to the enzyme activities obtained under standard conditions of the assay.

‖ $P < 0.025$, when compared to the enzyme activities obtained under standard conditions of the assay.

¶ $P < 0.05$, when compared to the enzyme activities obtained under standard conditions of the assay.

tyrosine hydroxylase, it is reasonable to expect that exposure of crude extracts containing tyrosine hydroxylase, activated by optimal K^+-depolarization to phosphorylating conditions, should produce no further or at least only a small increase in the activity of the enzyme. However, as shown in Table 2, the addition of Mg^{2+}, ATP and cAMP to tyrosine hydroxylase isolated from striatal slices incubated in 100 mM K^+ resulted in a further significant increase in enzyme activity. In fact, the absolute increase in tyrosine hydroxylase activity produced by exposure of the enzyme to phosphorylating conditions in this last experimental situation was slightly higher when compared to the absolute increase in enzyme activity resulting after adding Mg^{2+}, ATP and cAMP to enzyme obtained from slices incubated in normal KRP media (Table 2, third column). Suprisingly, exposure to phosphorylating conditions also significantly increased the activity of tyrosine hydroxylase in homogenates obtained from striatal slices incubated in the presence of optimal concentrations of dBcAMP (10 mM).

In summary, the observations reported here, albeit necessarily indirect and with the limitation of being obtained with crude enzyme preparations, do not support the conclusion that K^+ depolarization leads to an activation of tyrosine hydroxylase primarily via a cAMP-dependent phosphorylation process. Our findings offer no alternative explanation for the activation of tyrosine hydroxylase caused by neuronal depolarization. However, reports from this and other laboratories have emphasized the role that Ca^{2+} might play in this activation. Calcium addition has been shown to activate soluble tyrosine hydroxylase in a manner similar to that caused by neuronal depolarization [14,22,23] and the K^+-depolarization-induced activation of tyrosine hydroxylase appears to exhibit a calcium requirement [24]. Unexpectedly, our findings also suggest that dBcAMP activates tyrosine hydroxylase in striatal slices primarily by mechanisms other than cAMP-dependent phosphorylation. Dibutyryl cAMP could produce this activation through mobilization of intraneuronal Ca^{2+} compartments. Further experimentation with more purified enzyme preparations of activated tyrosine hydroxylase is needed in order to provide more definite answers to the above questions.

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Biologic properties of three anthracyclines as a function of lipophilicity

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Adriamycin and daunorubicin are well-known anthracycline antibiotics which inhibit growth of animal tumors [1] and are useful in the clinic [2]. Carminomycin [3] is a structural analog of daunorubicin, in which the —OCH_3 substituent on the C-4 position (ring 1) is replaced by —OH.

Accumulation of the anthracyclines is believed to involve a "leak and pump" system [4, 5]; enhanced drug exodus is one mode of drug resistance [5–7]. Once inside the cell, the