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TYROSINE HYDROXYLASE REGULATION IN RAT STRIATAL AND OLFACTORY TUBERCLE SLICES—I

ACTIVATION INDUCED BY EXPOSURE TO A CALCIUM-FREE AND HIGH-MAGNESIUM MEDIUM

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Abstract—Slices from rat corpus striatum and olfactory tubercle were incubated for 15 min at 37° in Krebs–Ringer phosphate (KRP) medium or Ca^{2+}-free KRP medium both in the presence and absence of high Mg^{2+} (12 mM). Tyrosine hydroxylase activity and kinetic parameters were determined in the 20,000 *g* supernatant fraction prepared from slices. The omission of Ca^{2+} from the incubation medium resulted in a moderate increase in the activity of striatal tyrosine hydroxylase, assayed in the presence of subsaturating concentrations of tyrosine and pterin cofactor, and a significant increase in the activity of tyrosine hydroxylase isolated from olfactory tubercle slices. The presence of Mg^{2+} (12 mM) in the Ca^{2+}-free KRP medium produced a further increase in the activity of the enzyme isolated both from striatal and olfactory tubercle slices. The per cent of stimulation of enzyme activity induced by incubating the slices in a Ca^{2+}-free, high-Mg^{2+} KRP medium was maximal when assayed at pH 7.0. The Mg^{2+}-induced activation of tyrosine hydroxylase was antagonized by increasing the Ca^{2+} concentration (3.75 to 15.0 mM) in the medium in which the slices were incubated. The direct addition of Mg^{2+} (5–20 mM) to striatal and olfactory tubercle homogenates also resulted in an increase in the activity of tyrosine hydroxylase. The addition of high concentrations of Ca^{2+} (10 mM) to the homogenates also resulted in an increase in enzyme activity but this effect was additive to that produced by Mg^{2+} (10 mM). Incubation of striatal slices in a Ca^{2+}-free, high-Mg^{2+} KRP medium produced changes in the kinetic properties of tyrosine hydroxylase. The apparent K_m of the enzyme for 6-methyl-5,6,7,8-tetrahydropterine HCl (6-MPH_4) was decreased significantly from 0.85 to 0.40 mM, with no significant change in the V_{max}. However, the K_i of the enzyme for dopamine (DA) was unchanged. Magnesium ions (12 mM) added to KRP-high K^+ (55 mM) medium blocked the activation of tyrosine hydroxylase induced by K^+-depolarization of striatal slices. However, Mg^{2+} (12 mM) addition to the incubation medium did not block but actually further increased the tyrosine hydroxylase activation that results after incubating striatal slices in a KRP medium enriched with dibutyryl cAMP (1 mM). Moreover, the stimulating effect on tyrosine hydroxylase observed when assays were conducted in the presence of Mg^{2+}, ATP and cAMP remained unchanged in homogenates prepared from slices previously incubated in a Ca^{2+}-free, high-Mg^{2+} KRP medium. The results reported in this work clearly demonstrate that a kinetic activation of tyrosine hydroxylase in dopaminergic nerve terminals can occur under conditions of diminished extracellular Ca^{2+} and blockade of transmitter release. Cyclic AMP does not seem to play a role in the tyrosine hydroxylase activation induced by incubation of slices in Ca^{2+}-free and high-Mg^{2+} medium. The results support the hypothesis that the increase in DA biosynthesis observed in dopaminergic neurons after inhibition of impulse flow may result primarily as a consequence of a diminished entrance of Ca^{2+} into the nerve terminal.

Several studies have now demonstrated that inhibition of firing in the nigro-neostriatal dopamine pathway induced pharmacologically or by a mechanical lesion is followed by an immediate and rapid accumulation of dopamine (DA) in the neostriatum [1–6]. A similar increase in DA has also been observed in the nucleus accumbens and olfactory tubercle after impulse flow is blocked in the dopaminergic afferents originating in the ventral mesencephalic tegmentum [7]. An analogous augmentation in the steady state levels of norepinephrine (NE) [8] or serotonin [9] is not observed when impulse flow in these respective neuronal systems is interrupted either pharmacologically or by electrothermic lesions. The rapid accumulation of DA seen after pharmacological or mechanical interruptions of impulse flow in the nigro-neostriatal pathway is explainable in part by a marked acceleration in dopamine biosynthesis. Treatments which abolish impulse flow enhance the conversion of labeled tyrosine into DA and stimulate the accumulation of dihydroxyphenylalanine (DOPA) after inhibition of DOPA decarboxylase [4, 10]. These latter results suggest that a cessation of impulse flow accelerates DA synthesis by increasing tyrosine hydroxylase activity. In fact, when impulse flow is blocked by administration of gamma-butyrolactone (GBL) or by placement of an electrothermic lesion in the nigro-neostriatal pathway, the activity of tyrosine hydroxylase measured *in vitro* in the presence of subsaturating concentrations of tyrosine and pterin cofactor was increased significantly [11]. In addition, the kinetic properties of the isolated enzyme were altered. Tyrosine

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hydroxylase obtained from the neostriata of GBL-treated rats or rats with nigral lesions displayed a significant increase in its affinity for both substrate and pterin cofactor and a significant decrease in its affinity for the end product inhibitor DA. Thus, a blockade of impulse flow in dopaminergic neurons may lead to a change in the physical properties of pre-existing tyrosine hydroxylase molecules which renders the enzyme less susceptible to end product inhibition and increases transmitter biosynthesis *in vivo*. These biochemical changes can be reversed if impulse flow is restored by electrical stimulation of the nigrostriatal pathway [12].

The precise molecular mechanism or event which triggers an alteration in the kinetic properties of dopaminergic tyrosine hydroxylase in response to a cessation of impulse flow in dopaminergic neurons, making the enzyme less susceptible to inhibition by endogenous DA, is at present unknown. Studies previously conducted in brain slices suggested a possible answer to this question [13]. In these studies the formation of DA from tyrosine in slices of rat striatum incubated in Krebs–Ringer phosphate medium was enhanced when calcium ions were removed from the medium. Thus, it appeared that CA^{2+} removal produced changes in striatal slices which led to an increase in dopamine formation. Moreover, under certain experimental conditions it was shown that the addition of calcium and of the calcium chelator EGTA to high-speed supernatant fractions caused, respectively, an inhibition and a stimulation in the activity of striatal tyrosine hydroxylase [14, 15]. These *in vitro* results suggested that changes in Ca^{2+} fluxes occurring in the dopaminergic nerve terminals may alter tyrosine hydroxylase activity *in vivo*. Accordingly, it was postulated that a blockade of impulse flow in the nigro-neostriatal pathway would prevent depolarization of the DA-containing nerve terminals in the neostriatum and the accompanying influx of Ca^{2+} [11]. This reduction in Ca^{2+} mobilization may activate tyrosine hydroxylase by changing the physical properties of preformed enzyme molecules or by initiating the formation of an enzyme activator.

In an attempt to test the above hypothesis, we have examined the specific activity and kinetic properties (K_m and V_{max}) of tyrosine hydroxylase isolated from brain slices previously exposed to experimental conditions which are believed to modify transmembrane calcium fluxes and reduce intraneuronal calcium concentrations. The absence of external Ca^{2+} has been reported to increase the efflux of Ca^{2+} from rat brain synaptosomal fractions and to decrease the concentration of intra-axoplasmic free calcium present in the squid giant axon [16, 17]. Also, a number of studies indicate that the lack of external Ca^{2+} in the incubation medium can affect some cellular functions, such as transmitter release and activation of adenylate cyclase [18, 19]. These same effects are produced by high external Mg^{2+} (12–24 mM). A high concentration of Mg^{2+} ions in the medium may very well modify the translocation of calcium through different intraneuronal compartments, thereby changing intracompartmental calcium concentrations. This paper is an account of a study in which we have examined the activity and kinetic characteristics of tyrosine hydroxylase isolated from striatal and olfactory tubercle slices previously incubated in either calcium-free or high-magnesium media.

MATERIAL AND METHODS

Preparation and incubation of rat striatal and olfactory tubercle slices. Tissue slices (0.18 mm in thickness) were prepared with a Sorvall tissue chopper, from the striatum and olfactory tubercles of adult, male Sprague–Dawley rats. Striatal and olfactory tubercle slices, weighing 30–40 mg and 10 mg, respectively, were incubated for 15 min at 37° in 5.0 ml of prewarmed Krebs–Ringer phosphate (KRP), pH 7.4, or in Ca^{2+}-free KRP, pH 7.4, saturated with 100% O_2 and in the absence and presence of high Mg^{2+} (12 mM). At the end of the incubation period, slices were separated from the medium by passage under vacuum through membrane filters (Schleicher and Schuell, No. 740-E) which contained a nylon mesh top (pore size = 35 μm). This procedure permitted a complete and rapid (2 sec) separation of slices from medium. The nylon mesh containing the slices was immediately frozen on dry ice and stored at −70° until the time of tyrosine hydroxylase assay. Homogenates prepared from slices of striata and olfactory tubercles were either assayed singly or pooled for determination of tyrosine hydroxylase kinetics.

Tyrosine hydroxylase assay. At the time of the assay, frozen striatal and olfactory tubercle slices were homogenized in 20 vol. of ice-cold 0.02 M Tris–acetate buffer, pH 7.0, and centrifuged at 20,000 *g* for 30 min at 4°. The supernatant fraction served as the source of soluble tyrosine hydroxylase. Tyrosine hydroxylase was assayed according to the method of Shiman *et al.* [20], with minor modifications. In this procedure, the production of ^3H_2O from L-[3,5-^{3}H]tyrosine is used as a measure of the amount of L-3,4-dihydroxyphenylalanine formed. The reaction was carried out in a total volume of 0.3 ml; 0.05 ml of striatal enzyme or 0.1 ml of olfactory tubercle enzyme added to a reaction mixture containing 15 μmoles morpholinopropane sulfonic acid (MOPS) buffer, pH 7.0; 3700 units of catalase; 0.01 ml (0.22 mg protein) of partially purified sheep liver dihydropteridine reductase; 0.15 μmole NADPH; and 0.03 μmole tetrahydrobiopterin (BH_4). In some enzyme assays, 0.075 μmole DL-6-methyl-5,6,7,8-tetrahydropterine HCl (6-MPH_4) was used instead of BH_4. After a 5-min preincubation at 37°, the reaction was initiated by the addition of 1.5 μCi L-[3,5-^{3}H]tyrosine (1 Ci/m-mole) in a volume of 0.05 ml. The [^{3}H]tyrosine was purified previously according to the method of Coyle [21] and evaporated to dryness just prior to use. After a 30-min incubation period at 37°, the reaction was stopped by the addition of 0.2 ml of 10% trichloroacetic acid (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 [22]. Protein was determined according to the method of Lowry *et al.* [23] using bovine serum albumin as a standard, and tyrosine hydroxylase activity was expressed as pmoles DOPA formed/mg of protein/min.

Kinetics were determined on the linear portion of the time course and protein concentration curves. Estimations of K_m for 6-MPH_4 were performed according to the method of Lineweaver and Burk [24] using a saturating concentration of tyrosine (0.1 mM) and six different 6-MPH_4 concentrations. The K_i values of DA were determined by the method of Dixon [25] at three

Table 1. Tyrosine hydroxylase activity in striatal slices previously incubated in Ca^{2+}-free and high-Mg^{2+} (12 mM) media

Treatment*	Tyrosine hydroxylase activity† (pmoles DOPA/mg protein/min)	Increase above KRP controls (%)
KRP	8.0 ± 0.6	0
Ca^{2+}-free KRP	10.2 ± 1.2	27.5
Ca^{2+}-free KRP + EGTA (1×10^{-4} M)	10.1 ± 0.4‡	26.3
Ca^{2+}-free, high-Mg^{2+} KRP	14.3 ± 0.4§,‖	78.8

*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 frozen in dry ice and subsequently homogenized and assayed for tyrosine hydroxylase.

† Tyrosine hydroxylase activity was determined in the 20,000 *g* supernatant fraction. Results are expressed as the mean ± S.E.M. of six different experiments (assayed in triplicate). Assays were conducted at pH 7.0 in the presence of 5 μM tyrosine and 100 μM BH_4.

‡ $P < 0.05$, when compared to normal KRP control.

§ $P < 0.001$, when compared to normal KRP control.

‖ $P < 0.01$, when compared to Ca^{2+}-free KRP.

6-MPH_4 concentrations. Data were analyzed using a paired *t*-test.

Solutions and chemicals. The Krebs–Ringer phosphate used had the following composition: NaCl, 128 mM; KCl, 4.8 mM; $CaCl_2$, 0.75 mM; $MgSO_4$, 1.2 mM; glucose, 16 mM; and Na_2HPO_4, 16 mM at pH 7.4. KRP-high Mg^{2+} (12 mM) and KRP-high K^+ (55 mM) were made by replacing proportions of NaCl with equimolar amounts of $MgSO_4$ and KCl respectively. Krebs–Ringer MOPS (KR MOPS), pH 7.4, was made by replacing Na_2HPO_4 with equimolar amounts of morpholinopropane sulfonic acid.

Dihydropteridine reductase was purified from sheep liver through the first ammonium sulfate fractionation according to the method of Kaufman [26]. An excess of reductase was used in the assay.

Morpholinopropane sulfonic acid, NADPH, cyclic AMP, dibutyryl cyclic AMP and ultrapure Tris were obtained from the Sigma Chemical Co., St. Louis, MO. Catalase was purchased from Boehringer-Mannheim, Indianopolis, IN, 6-MPH_4 from CalBiochem, La Jolla, CA, and L-[3,5-H^3]tyrosine (40 Ci/m-mole) was supplied by Amersham, Arlington Heights, IL.

RESULTS

Incubation of striatal slices in calcium-free KRP for 15 min resulted in a moderate increase in the activity of tyrosine hydroxylase found in the high speed supernatant fraction of homogenates prepared from the slices. A 30 per cent increase in activity was observed compared to the activity of tyrosine hydroxylase of striatal

Table 2. Tyrosine hydroxylase activity in olfactory tubercle slices previously incubated in Ca^{2+}-free and high-Mg^{2+} (12 nM) media

Treatment*	Tyrosine hydroxylase activity† (pmoles DOPA/mg protein/min)	Increase above KRP controls (%)
KRP	7.1 ± 0.3	0
Ca^{2+}-free KRP	10.7 ± 0.8‡	51
High-Mg^{2+}-KRP	13.3 ± 0.3§	87
Ca^{2+}-free, high-Mg^{2+} KRP	15.8 ± 1.1‖	122

* Olfactory tubercle slices were prepared by means of a Sorvall tissue chopper and incubated for 15 min at 37° in the various media. After the slices were separated from the medium, frozen on dry ice and subsequently homogenized and assayed for tyrosine hydroxylase.

† Tyrosine hydroxylase activity was determined in the 20,000 *g* supernatant fraction. Results are expressed as the mean ± S.E.M. of five different experiments assayed in triplicate. Assays were conducted at pH 7.0 in the presence of 5 μM tyrosine and 100 μM BH_4.

‡ $P < 0.01$, when compared to normal KRP control.

§ $P < 0.025$, when compared to Ca^{2+}-free KRP.

‖ $P < 0.01$, when compared to Ca^{2+}-free KRP; not significantly different when compared to high-Mg^{2+} KRP.

slices incubated in normal KRP media (Table 1). However, this increase reached statistical significance only when EGTA (1×10^{-4} M) was added to the Ca^{2+}-free medium. Increasing the magnesium concentration present in the Ca^{2+}-free KRP medium to 12 mM resulted in a further augmentation in the activity of tyrosine hydroxylase isolated from the striatal slices. In fact, this last condition produced an increase in enzyme activity of about 80 per cent over the activity found in striatal slices incubated in normal KRP (Table 1).

More striking effects were observed when olfactory tubercle slices were used instead of striatal slices (Table 2). Incubation of olfactory tubercle slices in Ca^{2+}-free KRP resulted in a significant increase (51 per cent) in the activity of tyrosine hydroxylase isolated from the slices. The presence of high Mg^{2+} (12 mM) in the Ca^{2+}-free KRP produced a further increase to 122 per cent in the activity of tyrosine hydroxylase isolated from olfactory tubercle slices. In fact, even the presence of Mg^{2+} (12 mM) in normal KRP was enough to produce an 87 per cent increase in the activity of tyrosine hydroxylase (Table 2). However, no significant difference was detected between the tyrosine hydroxylase activity obtained from olfactory tubercle slices incubated in high-Mg^{2+} KRP and those incubated in Ca^{2+}-free, high-Mg^{2+} KRP (Table 2).

Figure 1 shows the effect of varying the pH of the assay media on the activity of tyrosine hydroxylase isolated from striatal and olfactory tubercle slices previously incubated both in normal KRP and Ca^{2+}-free, high-Mg^{2+} (12 mM) KRP. In this experiment, the enzyme present in the high speed supernatant fraction of homogenates prepared from the slices was assayed through a pH range of 5.5 to 8.0. Tyrosine hydroxylase isolated from slices incubated in Ca^{2+}-free, high-Mg^{2+} KRP showed a pH profile similar to that of the enzyme isolated from slices incubated in normal KRP (Fig. 1). Moreover, the enzyme isolated from either striatal or olfactory tubercle slices showed a similar pH profile. Maximal enzyme activity was found at pH 5.8 to 6.0 in all cases. Baseline enzyme activities and the stimulation induced by Ca^{2+}-free, high-Mg^{2+} media started to decline rapidly at pH values higher than 7.2 (Fig. 1). However, the per cent stimulation of enzyme activity induced by incubating either striatal or olfactory tubercle slices in a Ca^{2+}-free, high-Mg^{2+} KRP medium was maximal and close to 100 per cent when assayed at around pH 7.0 as compared to 40–50 per cent at pH 5.8 to 6.0 (Fig. 1). Therefore, most of the experiments reported in this paper have been carried out at an assay pH of 7.0.

High concentrations of SO_4^{2-} ions (>1.0 M) have been reported to activate canine hypothalamic tyrosine hydroxylase [27]. Since Mg^{2+} is present in the incubation media as $MgSO_4$, the possibility existed that the activation induced by Mg^{2+} in slices and reported above (Tables 1 and 2) is not due to the Mg^{2+} moiety of the molecule but to the SO_4^{2-} moiety. In order to examine this possibility, the tyrosine hydroxylase activity was determined in striatal slices previously incubated in a KRP medium containing Na_2SO_4 (12 mM). The presence of SO_4^{2-} ions (12 mM) in the incubation medium

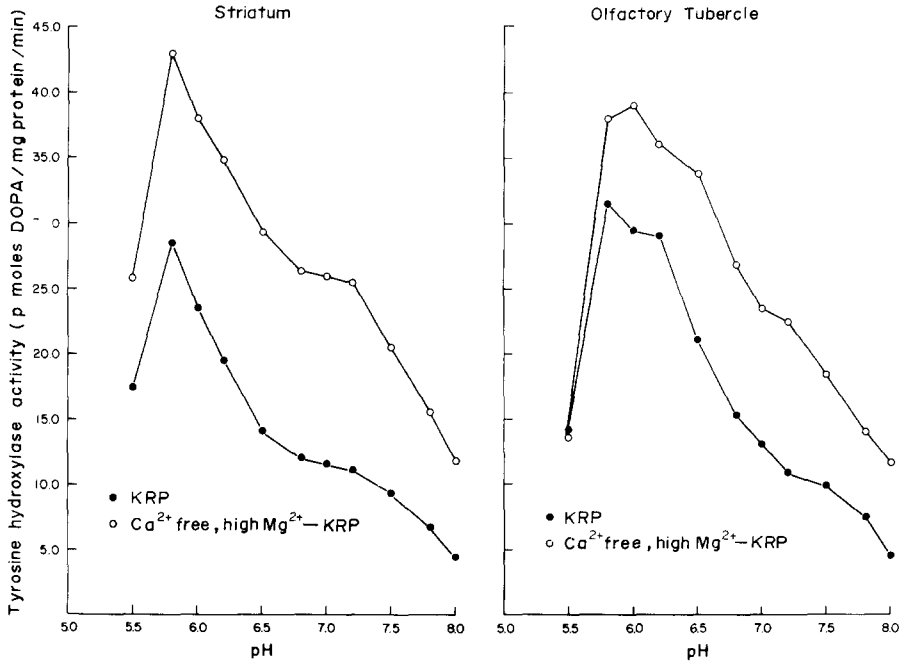

Fig. 1. Measurement of pH dependency of tyrosine hydroxylase isolated from striatal and olfactory tubercle slices previously incubated in normal KRP and Ca^{2+}-free, high-Mg^{2+} (12 mM) KRP. Slices were prepared by means of a Sorvall tissue chopper and incubated for 15 min at 37° in either medium. Thereafter the slices were homogenized in 20 mM Tris–acetate buffer at pH 7.0. Tyrosine hydroxylase was assayed in the 20,000 *g* supernatant fraction at the pH illustrated using 50 mM morpholinoethanesulfonic acid buffer in the pH range of 5.5 to 7.0 and 50 mM morpholinopropanesulfonic acid buffer in the pH range of 6.8 to 8.0. Results represent the average of two different experiments, each assayed in triplicate. Assays were conducted in the presence of 10 μM tyrosine and 250 μM 6-MPH_4.

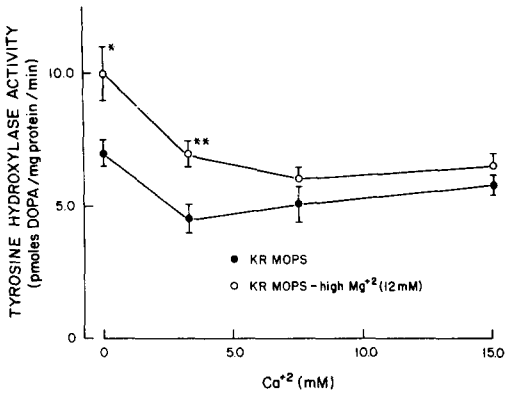

Fig. 2. Tyrosine hydroxcylase activity in rat striatal slices as a fuction of external Ca^{2+} concentration. Slices were prepared by means of a Sorvall tissue chopper and incubated for 15 min at 37° in KR MOPS or KR MOPS–high-Mg^{2+} (12 mM) media. Thereafter, slices were frozen in dry ice and subsequently homogenized in 20 mM Tris–acetate buffer at pH 7.0 and assayed for tyrosine hydroxylase. Tyrosine hydroxylase was determined in the 20,000 *g* supernatant fraction. Each point represents the mean ± S.E.M. of five different experiments (assayed in triplicate). Assays were conducted at pH 7.0 in the presence of 5 μM tyrosine and 100 μM BH_4. A single asterisk (*) indicates $P < 0.05$, and a double asterisk (**) indicates $P < 0.025$ when compared to representative controls with normal Mg^{2+}.

did not in itself produce any significant activation of the tyrosine hydroxylase. The activities obtained (mean ± S.E.M.; N = 5) at the end of the incubation period were, respectively, 9.9 ± 0.3 and 11.0 ± 0.6 pmoles DOPA/mg of protein/min for the control slices and slices incubated in high SO_4^{2-} (12 mM) containing media.

As shown in Fig. 2, the Mg^{2+}-induced activation of tyrosine hydroxylase enzyme is antagonized by increasing the Ca^{2+} concentration of the incubation media. High Mg^{2+} (12 mM) activities tyrosine hydroxylase only when Ca^{2+} is absent from the external medium or when external Ca^{2+} is present at concentrations up to 3.75 mM. However, the presence of higher Ca^{2+} concentrations (3.75 to 15.0 mM) abolishes the Mg^{2+}-induced activation of striatal tyrosine hydroxylase. The highest specific activity of tyrosine hydroxylase isolated from these tissues is obtained when the slices are incubated in Ca^{2+}-free medium. Enzyme activity then declines as a function of rising external Ca^{2+} concentrations and then slowly increases at concentrations above 3.75 mM. In these experiments, MOPS buffer was used instead of phosphate buffer in order to avoid the formation of calcium phosphate precipitates when increasing the external Ca^{2+} concentration of the incubation media.

Table 3 shows the direct effect of divalent cations upon tyrosine hydroxylase activity when added to the high speed supernatant fraction. The direction addition of magnesium ions (as $MgCl_2$ salt) to striatal and olfactory tubercle homogenates at concentrations up to 1 mM had no significant effect upon enzyme activity (data not shown). However, the addition of higher Mg^{2+} concentrations (5–20 mM) to the homogenates resulted in a 12–83 per cent increase in the activity of tyrosine hydroxylase (Table 3). This effect is not specific for magnesium since the addition of calcium (5–10 mM) to the homogenates also resulted in a 25–53 per cent increase in activity. Furthermore, the activation produced by the addition of magnesium (10 mM) to the striatal homogenates was not antagonized by the addition of calcium (10 mM) but, on the contrary, a summation of the activation effect was observed.

In order to learn more about the mechanisms involved in the increase in tyrosine hydroxylase activity which results from incubating the slices in different media prior to enzyme isolation, the kinetic properties of tyrosine hydroxylase were determined after each treatment. Incubation of the striatal slices for 15 min in a Ca^{2+}-free, high-Mg^{2+} KRP medium resulted in a substantial change in the kinetic properties of the enzyme. The apparent K_m of tyrosine hydroxylase for 6-MPH_4 was decreased from 0.85 to 0.40 mM, with no significant change in the V_{max} (Table 4). However, under the experimental conditions used, the K_i of the enzyme for dopamine was not found to change in a

Table 3. Effect of divalent cations on striatal and olfactory tubercle tyrosine hydroxylase activity in high speed supernatant fraction

Tissue	Assay condition	Tyrosine hydroxylase activity* (pmoles DOPA/mg protein/min)	Per cent change
Striatum	Standard	2.0 ± 0.2	0
	Mg^{2+} (5 mM)	2.2 ± 0.2	12
	Mg^{2+} (10 mM)	2.8 ± 0.1	40
	Mg^{2+} (20 mM)	3.6 ± 0.4	83
	Ca^{2+} (5 mM)	2.5 ± 0.1	25
	Ca^{2+} (10 mM)	3.0 ± 0.1	53
	Mg^{2+} (10 mM), Ca^{2+} (10 mM)	3.7 ± 0.2	86
Olfactory tubercle	Standard	3.3 ± 0.3	0
	Mg^{2+} (10 mM)	4.6 ± 0.3	39
	Mg^{2+} (20 mM)	4.8 ± 0.2	45

* Tyrosine hydroxylase was determined in the 20,000 *g* supernatant fraction obtained from rat striatum and olfactory tubercle homogenates. Results are expressed as the mean ± S.E.M. of four determinations. Assays were carried out at pH 7.0 in the presence of 5 μM tyrosine and 100 μM BH_4.

Table 4. Tyrosine hydroxylase kinetics in striatal slices previously incubated in Ca^{2+}-free and high-Mg^{2+} (12 mM) media

Treatment*	K_m 6-MPH_4† (mM)	V_{max}† (pmoles DOPA/mg protein/min)	K_i DA† (μM)
KRP	0.85 ± 0.12	254 ± 35	3.0, 4.5
Ca^{2+} free, high-Mg^{2+} KRP	0.40 ± 0.01‡	237 ± 17	4.0, 7.0

* Striata were dissected out and slices prepared by means of a Sorvall tissue chopper. Incubation conditions in the various media were as described under Table 1.

† Striatal slices were homogenized in ice-cold 0.02 M Tris–acetate buffer, pH 7.0, containing Triton X-100 (0.2%, w/v), and tyrosine hydroxylase was assayed in the 20,000 *g* supernatant fraction. The K_m and V_{max} for 6-MPH_4 were determined by the method of Lineweaver and Burk [24] at a tyrosine concentration of 10^{-4} M and six 6-MPH_4 concentrations ranging from 1.5×10^{-3} to 1.25×10^{-4} M. Each value is the mean ± S.E.M. of the intercepts generated from three separate lines. The K_i for DA was determined by the method of Dixon [25] at five dopamine concentrations (5×10^{-5} to 10^{-5} M) and three 6-MPH_4 concentrations (5×10^{-4} to 1.25×10^{-4} M). The values represent the intercepts generated from two different experiments.

‡ $P < 0.05$, when compared to normal KPR control.

Ca^{2+}-free, high-Mg^{2+} medium (Table 4). Potassium depolarization of striatal slices also results in a marked activation of tyrosine hydroxylase isolated from the slices (Table 5). However, the addition of Mg^{2+} (12 mM) to the KRP-high K^+ (55 mM) media partially blocked the activation of tyrosine hydroxylase induced by K^+-depolarization.

The addition of optimal concentrations of dibutyryl cAMP (1 mM) to normal KRP medium prior to the isolation of tyrosine hydroxylase from striatal slices caused a significant increase in enzyme activity (Fig. 3). Dibutyryl cAMP (dBcAMP) caused about a 75 per cent increase ($P < 0.005$) in activity. Similarly, the addition of dBcAMP (1 mM) to a high-Mg^{2+} KRP medium also resulted in a significant 73 per cent increase ($P < 0.01$) in tyrosine hydroxylase activity when compared to the activity obtained from striatal slices previously incubated in a high-Mg^{2+} (12 mM) KRP medium (Fig. 3). As reported above (Table 2), the addition of magnesium (12 mM) to normal KRP prior to enzyme isolation also caused a significant (50 per cent) increase in enzyme activity (Fig. 3). When tyrosine hydroxylase prepared from slices previously incubated in normal KRP was assayed under conditions optimal for cAMP-induced phosphorylation, about a 100 per cent increase in enzyme activity (Table 6) was observed. This stimulating effect induced by optimal phosphorylating conditions still remained in homogenates prepared from slices incubated in a CA^{2+}-free, high-Mg^{2+} KRP medium. As shown in Table 6, addition of Mg^{2+} (10 mM), ATP (1 mM) and cAMP (10 μM) also caused an additional 100 per cent increase in tyrosine hydroxylase activity when added to homogenates prepared from slices incubated in a Ca^{2+}-free, high-Mg^{2+} -KRP media.

DISCUSSION

The results described in this paper demonstrate that an increased enzyme activity is observed when tyrosine hydroxylase, prepared from striatal and olfactory tubercle slices incubated in a Ca^{2+}-free/high-Mg^{2+} medium, is assayed *in vitro* in the presence of subsaturating concentrations of tyrosine and pterin cofactor (Tables 1 and 2). It is possible to demonstrate this increase after manipulations which involve freezing, thawing

Table 5. Effect of high Mg^{2+} (12 mM) on K^+-depolarization-induced activation of striatal tyrosine hydroxylase activity

Treatment*	Tyrosine hydroxylase activity† (pmoles DOPA/mg protein/min)
KRP	5.7 ± 0.4
KRP-high K^+ (55 mM)	16.3 ± 1.0
KRP-high K^+ (55 mM), Mg^{2+} (12 mM)	13.1 ± 0.6‡

* Striata were dissected out and slices were prepared by means of a Sorvall tissue chopper. Incubation conditions in the various media were as described under Table 1.

† Striatal slices were homogenized and tyrosine hydroxylase was assayed in the 20,000 *g* supernatant fraction. Results are expressed as the mean ± S.E.M. of four different experiments (assayed in triplicate). Assays were conducted in the presence of 5 μM tyrosine and 100 μM BH_4.

‡ $P < 0.05$, when compared to KRP-high K^+ (55 mM).

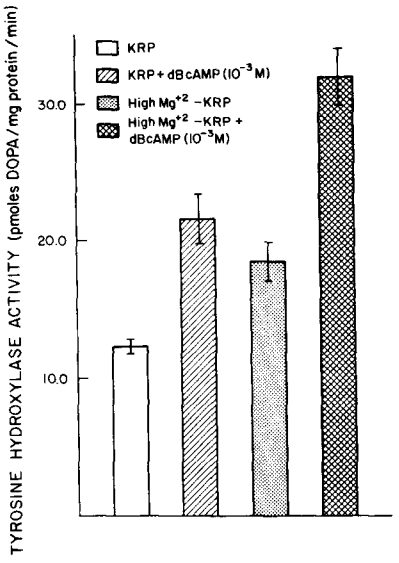

Fig. 3. Effect of dB cAMP on the Mg^{2+}-induced activation of striatal tyrosine hydroxylase. Striatal slices were incubated for 15 min at 37° in KRP and high-Mg^{2+} KRP either in the presence or the absence of 1 mM dB cAMP. Thereafter, the slices were frozen on dry ice and subsequently homogenized in 20 mM Tris–acetate buffer at pH 7.0 and assayed for tyrosine hydroxylase. Tyrosine hydroxylase was determined in the 20,000 *g* supernatant fraction. Each point represents the mean ± S.E.M. of four different experiments (assayed in triplicate). Assays were conducted at pH 7.0 in the presence of 10 μM tyrosine and 250 μM 6-MPH_4.

and homogenization of the slices. The enzyme activation appears to be mediated by a decrease in the apparent K_m of the enzyme for pterin cofactor. This activation of tyrosine hydroxylase is probably responsible for the accelerated DA formation observed when striatal slices are incubated in the absence of external Ca^{2+} ions [13]. In recent experiments, it has been demonstrated that incubation of olfactory tubercle slices in a Ca^{2+}-free and/or high-Mg^{2+} medium also results in an increase in the rate of dopamine biosynthesis [28]. Thus, activation and kinetic changes of tyrosine hydroxylase can occur in dopaminergic nerve terminals in the absence of appreciable transmembrane Ca^{2+} influx and in the presence of conditions that normally block DA release.

Potassium depolarization of dopaminergic nerve terminals has been reported to accelerate DA synthesis and to increase the activity of tyrosine hydroxylase [29]. This increase in tyrosine hydroxylase seemed to occur as a result of kinetic alterations in the enzyme similar to those reported in this work. However, absence of external calcium ions has been shown to block K^+-depolarization-induced activation of hippocampal tyrosine hydroxylase [30] and to prevent the K^+-induced acceleration of DA biosynthesis in olfactory tubercle slices [28]. We now report that high Mg^{2+} (12 mM) partially reduces the activation of striatal tyrosine hydroxylase induced by K^+-depolarization. In normal Krebs media high Mg^{2+} in a fashion similar to Ca^{2+}-chelating agents causes an activation of tyrosine hydroxylase. Therefore, the sequence of events which leads to tyrosine hydroxylase activation during K^+-depolarization of slices may not be the same as that leading to a kinetic activation of the enzyme after exposure to a CA^{2+}-free/high-Mg^{2+} medium.

Although the results reported in this paper indicate that incubation of slices in Ca^{2+} free/high external Mg^{2+} can result in an activation of tyrosine hydroxylase, the question still remains as to how this activation is brought about. As indicated in the introduction, the absence of external Ca^{2+} results in an increased Ca^{2+} efflux from ^{45}Ca-loaded synaptosomes prepared from rat brain [16] and in a fall in the internal ionized Ca^{2+} concentration present in the giant axon of the squid [17]. Moreover, *in vitro* studies have indicated that removal of calcium by the addition of the calcium chelator EGTA (50 μM) to soluble preparations of tyrosine hydroxylase caused a marked increase in the activity of the enzyme [15]. The activation of tyrosine hydroxylase produced by addition of EGTA to the incubation medium is in many ways similar to that reported in this work. Both treatments result in an enzyme which has an increased affinity for pterin cofactor. However, the addition of EGTA to the incubation medium also results in a dramatic decrease in affinity for DA. Under the experimental conditions of this work it was not possible to detect changes in the K_i for DA. It is possible that this is due to the different assay conditions used in these experiments. Catechols become

Table 6. Effect of phosphorylating conditions on the activity of Ca^{2+}-free and high-Mg^{2+}-induced activated tyrosine hydroxylase enzyme

Pretreatment*	Assay condition	Tyrosine hydroxylase activity† (pmoles DOPA/mg/min)
KRP	Standard	10.1 ± 0.8
KRP	Mg^{2+} (10 mM), ATP (1 mM), cAMP (10 μM)	20.5 ± 1.3‡
Ca^{2+}-free, high-Mg^{2+} KRP	Standard	16.0 ± 1.3§
Ca^{2+}-free, high-Mg^{2+} KRP	Mg^{2+} (10 mM), ATP (1 mM), cAMP (10 μM)	30.7 ± 3.4‖

* Striata were dissected out and slices were prepared by means of a Sorvall tissue chopper. Incubation conditions in the various media were as described under Table 1.

† Tyrosine hydroxylase was assayed as described under Table 1. Results represent the mean ± S.E.M. of five different experiments assayed in triplicate.

‡ $P < 0.001$, when compared to respective control assayed without Mg^{2+}, ATP and cAMP.

§ $P < 0.025$, when compared to respective KRP normal control.

‖ $P < 0.01$, when compared to respective control assayed without Mg^{2+}, ATP and cAMP.

much more potent inhibitors of tyrosine hydroxylase as the assay pH is raised [31], and the current experiments were carried out at pH 7.0 rather than at 6.0. The effects of EGTA on striatal tyrosine hydroxylase have not been documented by Lerner *et al.* [32]. However, this group did see an increase in tyrosine hydroxylase activity when using a G-25 Sephadex-treated enzyme. In summary, the previous experiments suggest that the activation of tyrosine hydroxylase reported in this work is brought about by a lowering in the internal Ca^{2+} concentration in the dopaminergic nerve terminals.

In the current experiments, the activation of tyrosine hydroxylase induced by incubation of striatal slices in a Ca^{2+}-free medium, whether in the absence or presence of EGTA, was never higher than 30 per cent (Table 1). In contrast, the activation of tyrosine hydroxylase observed in olfactory tubercles was always higher than 50 per cent (Table 2). Interestingly, the relative increase in dopamine biosynthesis induced by incubation of tissue slices in a Ca^{2+}-free medium is higher in olfactory tubercle than in the striatum [28]. Under these experimental conditions it may not be possible to lower the free intraneuronal Ca^{2+} concentrations sufficiently to affect biosynthesis. When high-Mg^{2+} concentrations were added to the Ca^{2+}-free medium an even greater activation of tyrosine hydroxylase was found (Tables 1 and 2). This raises the question of whether Mg^{2+} has a direct effect upon tyrosine hydroxylase or is acting indirectly through intracellular displacement of Ca^{2+} ions. We favor the latter possibility in view of the following arguments. First, the Mg^{2+}-induced activation of tyrosine hydroxylase in striatal slices is antagonized by increasing the external Ca^{2+} concentration of the medium. In fact the Mg^{2+} effect is seen only in the absence of external Ca^{2+} or in the presence of external Ca^{2+} concentrations up to 3.75 mM (Fig. 2). By contrast, the direct effect of Mg^{2+} upon tyrosine hydroxylase is not antagonized but actually is additive to that produced by adding Ca^{2+} ions to high-speed supernatant fractions (Table 3). Second, the activating effect that results by adding Mg^{2+} directly to the homogenate is observable only when using concentrations of 5–10 mM of this ion. According to Veloso *et al.* [33], the Mg^{2+} concentration in the cytoplasm is about 1 mM, of which 30–50 per cent is ionized Mg [34]. According to the Nernst equation, ionized Mg^{2+} in the cytoplasm would have to be about 100 to 200 times higher than the previous figure if the Mg^{2+} equilibrium potential were to equal the resting potential of the nerve cell membrane (−60 mV; cytoplasm negative). However, free Mg^{2+} in the cytoplasm is maintained between 0.5 and 1 mM by an outward transport in exchange of an influx of sodium ions [34] and by numerous intracellular organic ligands such as ATP, aspartate, glutamate, citrate and so on [35]. It seems very unlikely that the use of 12 mM external Mg^{2+} concentrations could result then in 10 or even 5 mM internal ionized Mg^{2+} concentrations. Moreover, even the addition of 5–10 mM Mg^{2+} directly to the homogenates produced an activation of tyrosine hydroxylase enzyme which was less than that observed after incubating the slices in 12 mM external Mg^{2+} (compare Table 2 vs Table 3).

As indicated above, it seems that Mg^{2+}-induced activation of the enzyme is indirect, i.e. movement of intraneuronal Ca^{2+} and lowering of the free Ca^{2+} concentration in the cell may be linked to this activation. The presence of high external Mg^{2+} may antagonize the influx of Ca^{2+} ions which may have leaked out from the nerve terminals. In this regard other cells such as mammalian erythrocytes or squid axon have been shown to possess an ATP-dependent Ca^{2+} efflux which requires the presence of Mg^{2+} in order to be activated [36]. Moreover, Kendrick *et al.* [37] have presented evidence for an ATP-dependent Ca^{2+} transport system located within pre-synaptic nerve terminals which may help to regulate internal ionized Ca^{2+} concentrations. The ATP-promoted Ca^{2+} transport seems to involve a Mg^{2+}-dependent ATPase, possibly located within the synaptic vesicle, and it is totally abolished in the absence of Mg^{2+}. Possibly, by acting through these transport systems, a high external concentration of Mg^{2+} could lower the internal ionized calcium concentration and further enhance the activation of tyrosine hydroxylase, thus explaining the results described in Tables 1 and 2.

Recently it has become appreciated that the addition of cAMP together with ATP, Mg^{2+} and protein kinase to preparations of tyrosine hydroxylase *in vitro* brings about a change in the physical properties of the enzyme. Such changes cause tyrosine hydroxylase to have an increased affinity for pterin cofactor and a decreased affinity for inhibitors with a catechol nucleus [38–40]. In experiments using either striatal slices or synaptosomes, both depolarization and exposure to dibutyryl cyclic AMP result in an increase in the activity and changes in the kinetic properties of tyrosine hydroxylase prepared from the treated tissue [29, 38, 39, 41]. It has been postulated that either: (1) cAMP accumulation and a consequent activation of a cAMP-dependent protein kinase may result in phosphorylation of tyrosine hydroxylase, or (2) an intermediate activator might be responsible for the activation of tyrosine hydroxylase observed after depolarization. Thus, incubation of slices in Ca^{2+}-free or high-Mg^{2+} media could activate tyrosine hydroxylase by first stimulating the accumulation of cAMP and second by producing the appropriate phosphorylating conditions. However, Ca^{2+}-free/high-Mg^{2+}-induced activation of tyrosine hydroxylase remained fully effective even in the presence of optimal dBcAMP concentrations in the incubation medium (Fig. 3) Moreover, addition of cAMP, Mg^{2+} and ATP to homogenates containing tyrosine hydroxylase prepared from free-Ca^{2+}/high-Mg^{2+}-treated slices maintained its effectiveness in further activating tyrosine hydroxylase (Table 6). In addition, the pH profile of tyrosine hydroxylase activated by incubation of striatal slices in dibutyryl cAMP (J. Simon and R. H. Roth, unpublished results) differs substantially from the pH profile of the enzyme activated by incubation of slices in high-Mg^{2+}-containing media. The pH optimum of the dibutyryl cAMP-activated enzyme is 7.0 to 7.2 while the pH optimum of the enzyme prepared from slices incubated in high Mg^{2+} is 6.0, similar to the control enzyme. These experiments, as well as the observations cited above, although not conclusive, provide evidence suggesting that cAMP and phosphorylation do not play a role in the tyrosine hydroxylase activation induced by incubation of slices in Ca^{2+} -free and high-Mg^{2+} media. Although these data tend to discredit the hypothesis that cAMP might be involved in this activation process, the present experiments do not provide an alternative explanation as to how a drop in ionized Ca^{2+} in the

axoplasm could effect changes in the kinetic properties of this enzyme.

Incubation of brain slices in Ca^{2+}-free and high-Mg^{2+} media results in an increase in the activity as well as changes in the kinetic properties of tyrosine hydroxylase. These results thus support the hypothesis that the increase in DA biosynthesis observed in dopaminergic neurons after inhibition of impulse flow may occur primarily as a consequence of a diminished entrance of Ca^{2+} into the nerve terminal. Finally, these results demonstrate that a kinetic activation of tyrosine hydroxylase and an increase in transmitter synthesis in dopaminergic nerve terminals occur under conditions of diminished extracellular Ca^{2+} and a blockade of transmitter release.

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