*Biochemical Pharmacology*, Vol. 35, No. 12, pp. 1929–1932, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

# $K^+$-DEPENDENT STIMULATION OF TYROSINE HYDROXYLATION IN STRIATAL SLICES: ROLE OF $Ca^{2+}$/CALMODULIN AND $Ca^{2+}$/PHOSPHOLIPID-DEPENDENT MECHANISMS

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(Received 20 December 1985; accepted 19 March 1986)

During periods of increased impulse flow in central dopaminergic neurons the physical properties of tyrosine hydroxylase (TH) are altered, resulting in activation of the rate-limiting step in DA biosynthesis (1-4). A similar activation can be produced *in vitro* by $K^+$ depolarization of striatal slices, although the nature of the kinetic changes accompanying this activation is controversial (5-7).

Calcium can produce an activation of soluble striatal TH characterized by a decrease in the apparent $K_m$ for pteridine cofactors and an increase in the $K_i$ for DA. This activation requires ATP and magnesium, indicating that it results from a $Ca^{2+}$-dependent phosphorylation process rather than a direct interaction of $Ca^{2+}$ with the enzyme (8). Incubation of striatal tissue under conditions which promote cAMP-dependent phosphorylation of TH also results in a kinetic activation of the enzyme. However, evidence is accumulating to suggest that $Ca^{2+}$-dependent, rather than cAMP-dependent, phosphorylation events are responsible for TH activation induced by depolarization (6,7,9,10).

Both $Ca^{2+}$/calmodulin-dependent protein kinase II and $Ca^{2+}$/phospholipid-dependent kinase (protein kinase C) have been found to phosphorylate and activate purified TH *in vitro* (11-13). Recent evidence from studies conducted in striatal slices suggests an involvement of a calmodulin-dependent mechanism (7), but the possible involvement of the protein kinase C system in the depolarization-induced activation of striatal TH has not been evaluated. In the present study, accumulation of DOPA after addition of a decarboxylase inhibitor to the incubation medium was used as an index of tyrosine hydroxylation in the slice (14), thereby providing a functional measure of *in situ* TH activity. Our results support the involvement of a $Ca^{2+}$/calmodulin dependent process in the depolarization-induced increase in striatal TH activity, and suggest that conditions which activate protein kinase C in other systems do not elicit an increase in tyrosine hydroxylation in striatal slices.

## MATERIALS AND METHODS

Male Sprague Dawley rats (250-300 g) obtained from Charles River Labs (Kingston, NY) were killed by decapitation. Striata were removed as previously described (15). DOPA accumulation in striatal slices was determined by the method of Wolf *et al.* (14). Briefly, slices (250 μm) were prepared using a Sorvall tissue chopper and transferred to vials containing 2 ml of preoxygenated Krebs-Ringer-MOPS (KRM, pH 7.4) consisting of the following: NaCl, 128 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; glucose, 11.1 mM; L-tyrosine, 0.1 mM; and morpholinopropane sulfonic acid (MOPS), 15.8 mM. $Ca^{2+}$-free conditions were achieved by replacing $CaCl_2$ with NaCl in order to maintain osmolarity. DOPA accumulation was also measured in $Ca^{2+}$-free medium containing ethylene glycol bis(beta-aminoethyl ether)-N,N'-tetraacetic acid (EGTA, 0.1 mM). The incubation vials were maintained at 37° in a shaking water bath with 95% $O_2$/5% $CO_2$ continually blown over the surface of the incubation medium. The aromatic amino acid decarboxylase inhibitor m-hydroxybenzylhydrazine (NSD-1015) was added following a 40-minute preincubation period (i.e. at t = 40). The final concentration of NSD-1015 was 200 μM; this concentration promoted maximal basal DOPA accumulation. In experiments examining $K^+$-stimulated DOPA formation, KCl (final concentration, 30 mM) was added

at t = 30 min. DOPA accumulation was allowed to proceed for 10 min after the addition of NSD-1015. Incubations were stopped (t = 50 min) by sonication immediately following the addition of 500 µl of 0.5 N perchloric acid containing alpha-methyl DOPA as an internal standard. Catechols were isolated from tissue and media as described previously (14). Amounts of DOPA and alpha-methyl DOPA in each sample (tissue + medium) were determined using HPLC with electrochemical detection (16). Protein content was determined as described by Bradford (17). W-7 (N-(6-aminohexyl)-5-chloro-1-napthalene-sulfonamide HCl) and TPA (12-O-tetradecanoylphorbol-13-acetate) were added as indicated in Fig. 1 and Table 1.

MOPS, NSD-1015, W-7, and TPA were obtained from the Sigma Chemical Co. (St. Louis, MO). Other reagents were obtained from commercial sources. NSD-1015 was dissolved in KRM, W-7 was dissolved in 0.1% acetic acid and diluted 1:2000 with KRM before use, and TPA was dissolved in dimethyl sulfoxide and diluted 1:10,000 with KRM before use. Vehicle controls were performed for all experiments and were not found to differ significantly from normal KRM controls. Experimental groups were compared using a two-way analysis of variance, and subsequent post-hoc analyses were performed when indicated using Tukey tests.

## RESULTS AND DISCUSSION

Incubation of striatal slices with the decarboxylase inhibitor NSD-1015 results in a linear accumulation of DOPA for at least 45 min (14). Exposure of striatal slices to high $K^+$ (30 mM) KRM for 10 min prior to addition of NSD-1015 induced a 61% increase in DOPA formation (Table 1). We initially examined whether this $K^+$-induced stimulation of tyrosine hydroxylation was dependent on the presence of extracellular $Ca^{2+}$. Basal DOPA formation in $Ca^{2+}$-free medium was found to be 36% greater than observed in normal KRM. However, exposure of slices to 30 mM $K^+$ produced no further enhancement of DOPA accumulation under $Ca^{2+}$-free conditions (Table 1), indicating that the stimulation of DOPA formation produced by high $K^+$ is $Ca^{2+}$-dependent. This finding is in agreement with the results of other studies in striatal slices (10) and synaptosomes (18).

Albert *et al.* (13) have reported that protein kinase C phosphorylates TH purified from PC12 cells, resulting in a kinetic activation of the enzyme. Similarly, protein kinase C has been demonstrated to phosphorylate TH purified from rat pheochromocytoma (19). Phorbol esters such as TPA have been shown to directly activate protein kinase C *in vivo* (20). Also, addition of TPA to PC12 cells produces an increase in tyrosine hydroxylation (21). Thus, if depolarization-induced activation of striatal TH is mediated by protein kinase C, we hypothesized that TPA may mimic the stimulation of DOPA formation produced by 30 mM $K^+$. However, exposure of striatal slices to 0.1 to 1.0 µM TPA for as long as 50 min failed to alter basal or $K^+$-stimulated DOPA accumulation (Table 1). This suggests that either activation of protein kinase C in itself is not sufficient to elicit increased tyrosine hydroxylation in striatal slices or TPA does not activate protein kinase C in striatal slices under our experimental conditions. Studies are in progress to determine the effect of TPA on protein phosphorylation in striatal slices in order to address this issue.

Yamauchi and Fujisawa (11) have demonstrated that rat brain TH is phosphorylated *in vitro* by $Ca^{2+}$-calmodulin dependent protein kinase II, although this results in enzyme activation only in the presence of an activator protein. Similarly, Vulliet *et al.* have described the phosphorylation of TH isolated from pheochromocytoma cells by a $Ca^{2+}$-calmodulin dependent protein kinase (19). We have used W-7, a potent calmodulin antagonist (22) which readily penetrates cell membranes, to investigate the involvement of $Ca^{2+}$/calmodulin dependent processes in the $K^+$-induced activation of TH. W-7 (0.5-50 µM) produced a dose dependent reduction in basal and $K^+$-stimulated DOPA accumulation after decarboxylase inhibition (Fig. 1). During the course of these studies similar results were reported by Hirata and Nagatsu (23).

The finding that W-7 can block $K^+$-stimulated DOPA formation in striatal slices is in accord with a recent report by El Mestikaway *et al.* (7) that W-7 prevents the increase in TH activity normally observed when the enzyme is obtained from striatal slices incubated in high $K^+$ medium.

Table 1. DOPA Formation in Striatal Slices

| Incubation conditions | DOPA Accumulation (% Control) 4.8 mM $K^+$ | 30 mM $K^+$ |
|---|---|---|
| KRM | 100 ± 11 (4) | 161 ± 15 ** (4) |
| $Ca^{2+}$-free KRM | 136 ± 4 ** (5) | 137 ± 8 ** (4) |
| $Ca^{2+}$-free KRM, EGTA (0.1 mM) | 137 ± 6 ** (5) | 131 ± 3 ** (5) |
| KRM, TPA (100 nM, 50 min) | 89 ± 9 (4) | ND |
| KRM, TPA (250 nM, 20 min) | 91 ± 2 (4) | ND |
| KRM, TPA (250 nM, 50 min) | 115 ± 15 (4) | 163 ± 12 ** (4) |
| KRM, TPA (1.0 μM, 50 min) | 107 ± 15 (4) | ND |
| $Ca^{2+}$-free KRM, W-7 (5.0 μM) | 145 ± 16 * (8) | ND |

Basal and $K^+$-stimulated DOPA formation were measured as described in Materials and Methods. DOPA accumulation was allowed to proceed for 10 min following the addition of NSD-1015. TPA was added either at the beginning of the incubation or 10 min prior to NSD-1015. Each value represents the mean ± SEM of the number of incubates indicated in parentheses, each consisting of one sliced striatum (** = $P < 0.01$, * = $P < 0.05$ relative to KRM control). DOPA value for KRM control = 16.5 ng/mg protein; ND = not determined.

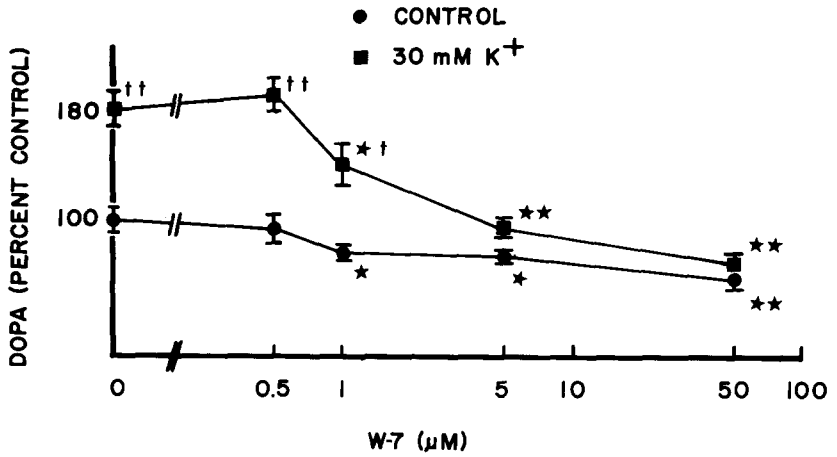


Fig. 1. Basal and $K^+$-stimulated DOPA accumulation in striatal slices were measured as described in Materials and Methods. W-7 (0.5-50 μM) was in the media at the start of incubation. In $K^+$-stimulated experiments KCl (final concentration, 30 mM) was added at t = 30 min. NSD-1015 was added at t = 40 min; the incubations were stopped at t = 50 min. Symbols indicate significant difference (†,* = $P < 0.01$; ††, ** = $P < 0.05$) from corresponding value obtained in the absence of W-7 (*) or $K^+$ (†).

In contrast to our results, these investigators did not observe a reduction in the basal activity of TH obtained from striatal slices incubated with W-7. This difference may reflect the different methods used to assess TH activity, i.e. DOPA accumulation *in situ* versus $^3H_2O$ formation *in vitro*. Interestingly, Lee *et al.* (24) observed that W-7 reduces the enhancement of TH phosphorylation in PC12 cells produced by elevated $K^+$.

In contrast to the inhibition of $K^+$-stimulation of TH observed in striatal slices, W-7 (5.0 μM) did not reduce significantly the enhancement of tyrosine hydroxylation observed in incubations carried out in $Ca^{2+}$-free media (Table 1). This suggests that the increase in DOPA accumulation caused by low extracellular calcium involves a mechanism different from that involved in $K^+$-stimulated DOPA accumulation.

In summary our results indicate that exposure to TPA, a treatment which activates protein kinase C in other systems, did not mimic $K^+$-depolarization in striatal slices since it did not alter tyrosine hydroxylation. Furthermore, addition of a calmodulin antagonist, W-7, inhibited the increase in tyrosine hydroxylation caused by $K^+$-depolarization. This suggests that depolarization-induced activation of TH may be mediated through a calmodulin-dependent process. Studies on the modulation of TH kinetics and phosphorylation by $Ca^{2+}$-dependent kinases in striatal slices are necessary to further elucidate the mechanisms underlying control of TH activity by neuronal depolarization.

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