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FURTHER STUDIES ON THE ROLE OF CALCIUM IN THE DEPOLARIZATION-INDUCED ACTIVATION OF TRYPTOPHAN HYDROXYLASE

EFFECT OF VERAPAMIL, TETRACAINE, HALOPERIDOL AND FLUPHENAZINE

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Abstract—Tryptophan hydroxylase becomes activated by a calcium-dependent process following depolarization of slices of rat brain stem in a potassium-enriched medium. The present study shows that the mechanism responsible for raising enzyme activity is sensitive to increasing concentrations of calcium in the depolarizing medium. Enzyme activity increased progressively as the calcium concentration in the medium was raised from 0.1 to 5.0 mM. The depolarization-induced activation was fully reversible and could be blocked by verapamil, a calcium channel blocking agent, tetracaine, a local anesthetic, and two antipsychotic drugs, haloperidol and fluphenazine, which bind calmodulin. Unlike verapamil and tetracaine, the antipsychotics appeared to have an intracellular site of action, for they blocked the increase in enzyme activity induced when brain stem slices were incubated in a calcium-free medium in the presence of a metabolic inhibitor, such as guanidine. This activation is presumed to be due to the increase in intracellular free calcium that occurs in the presence of such poisons. The inhibitory effects of haloperidol and fluphenazine on the depolarization-induced activation of tryptophan hydroxylase, taken together with published evidence that supernatant preparations of the enzyme are activated when incubated under phosphorylating conditions by a calcium–calmodulin-dependent process, suggest that calmodulin may mediate the calcium-dependent activation induced by depolarization.

Nerve stimulation increases 5-HT* synthesis *in vivo* [1, 2] through the enhanced conversion of tryptophan to 5-HTP by tryptophan hydroxylase [3] [tryptophan-5-monooxygenase, L-tryptophan, tetrahydropterin:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4], the rate-limiting enzyme in 5-HT synthesis. Although the mechanism mediating this effect *in vivo* has not been identified, *in vitro* studies with slices of rat brain have revealed that depolarization not only increases 5-HT synthesis [4–6] but also produces an activation of tryptophan hydroxylase that can be detected when kinetic measurements are made on supernatant fractions from homogenates of depolarized slices [4, 7, 8]. Both the increases in 5-HT synthesis in the brain slices and the alteration in enzyme activity share a requirement for calcium ions and can be blocked by omission of calcium from the incubation medium or its replacement with other divalent cations, such as magnesium or cobalt [4, 5, 7]. The fact that alterations in tryptophan availability make no contribution to the enhanced synthesis of 5-HT observed *in vivo* or in depolarized slices [2, 3, 5] suggests that a change in the kinetic behavior of tryptophan hydroxylase could mediate the effects of depolarization on 5-HT synthesis. This view is strengthened by recent studies on supernatant preparations of the enzyme showing that it can be activated under phosphorylating conditions [9–14] by a calcium–calmodulin-dependent process [13, 14] that may involve a calcium–calmodulin-dependent protein kinase [15].

The present studies were undertaken to characterize the depolarization-induced activation of tryptophan hydroxylase further and, in particular, to determine whether it shares properties with the stimulation-induced increase in 5-HT synthesis (e.g. frequency dependency, reversibility [2]) and the activation observed when supernatant preparations of the enzyme are incubated under phosphorylating conditions (e.g. reversibility, requirement for calmodulin [13, 14]). Some of the results presented here have already been communicated in preliminary form [16].

MATERIALS AND METHODS

The procedures used in this study have already been described in detail in two earlier reports [7, 8] and will, therefore, only be outlined briefly here. Male Sprague–Dawley rats (Flow Laboratories, Rockville, MD) weighing 150–200 g were decapitated, and the brain stem (diencephalon, midbrain and medulla-pons) was removed, chilled, and cut into 250 μm slices with an MT-2 tissue chopper

* Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; 6-MPH$_4$, DL-6-methyl-5,6,7,8-tetrahydropterin; EGTA, ethylene glycol bis-(β-aminoethylether)-*N,N'*-tetraacetic acid; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; dibutyryl cyclic AMP, N^6,O^2-dibutyryl adenosine-3':5'-cyclic monophosphate; and cyclic AMP, adenosine-3':5'-cyclic monophosphate.

(Dupont Sorvall, Newtown, CT). Each sliced brain stem was incubated for 10 min or longer at 25° in 5 or 10 ml of oxygenated control (150 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Tris acetate buffer, pH 7.4) or experimental medium in which various modifications had been made or to which drugs or poisons had been added. The experimental media are described in detail in Results. At the end of the incubation period, the slices were separated from the medium by centrifugation (10,000 *g* for 10 min) at 4° in an RC-5 high speed refrigerated centrifuge (Dupont Sorvall), homogenized (1:1.5, w/v) in 0.05 M Tris acetate buffer at pH 7.4, and recentrifuged for 30 min at 39,000 *g*. DTT (final concentration, 2 mM) was added to the low speed supernatant fraction, which was then passed over a Sephadex G-25, course type (Pharmacia, Piscataway, NJ) column (22 × 1.3 cm) equilibrated with 0.05 M Tris acetate (pH 7.4) containing 2 mM DTT, at 7° (cold room temperature). The pink column effluent was used for the tryptophan hydroxylase assay which was a modification of the procedure of Friedman *et al.* [17]. This assay was carried out in the presence of 200 μM L-tryptophan and 50 μM synthetic reduced pterin cofactor, 6-MPH_4, which was regenerated by NADPH and endogenous dihydropteridine reductase. Details are given elsewhere [8]. The concentration of cofactor was subsaturating and thus these assay conditions permitted detection of enzyme activation. Each brain stem enzyme preparation was assayed in quintuplet or sextuplet (linearity of the reaction with time and protein was always checked) and the values were averaged. Results are the mean ± S.E.M. of values from three or more brain stem enzyme preparations, unless otherwise indicated, and are expressed as pmoles 5-HTP formed per mg protein per min or as percent control activity (100). Protein was determined by the method of Lowry *et al.* [18] using bovine serum albumin as standard.

The drugs used in this study were obtained from the following sources: verapamil, Knoll Pharmaceutical Co., Whippany, NJ; haloperidol, McNeil Pharmaceutical Co., Spring House, PA; fluphenazine, E. R. Squibb & Sons, Inc., Princeton, NJ; and tetracaine, Mann Research Laboratories, NY, a gift of Dr. Ronald Rubin of the Medical College of Virginia.

RESULTS

Determination of the optimum potassium concentration in the slice incubation medium. Depolarization of slices of rat brain in 66 mM KCl for 10 min at 25° increased the activity of tryptophan hydroxylase by 60–70% when the enzyme was assayed under standard conditions (50 μM 6-MPH_4, 200 μM L-tryptophan) in a low speed supernatant fraction prepared from the brain stem slices [7]. Figure 1 shows that under these incubation conditions there was a progressive increase in enzyme activity as the concentration of KCl in the medium was increased from 6 mM in unmodified medium to 66 mM KCl. A further increase in the concentration of KCl did not increase enzyme activity under the conditions of these experiments. Therefore, the present studies on

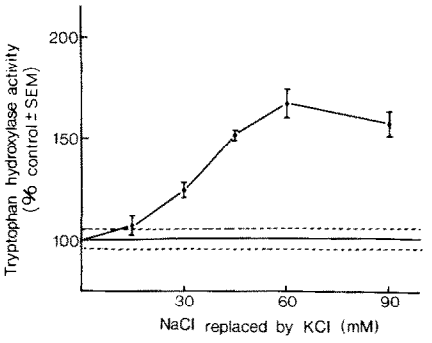

Fig. 1. Increase in the activity of tryptophan hydroxylase prepared from brain stem slices incubated in media in which increasing concentrations of NaCl were replaced with equimolar KCl. The final concentrations of KCl were actually 6 mM higher than the values indicated on the abscissa since the control medium already contained 6 mM KCl. Incubations were carried out for 10 min at 25°. Results, expressed as percent of control activity, are the mean ± S.E.M. of values from three brain stem slice preparations for each condition tested, except for control and 66 mM KCl where N = 26. Control enzyme activity (100) was 234 ± 8 pmoles 5-HTP per mg protein per min. The level of significance of the difference between the activity of enzyme from depolarized and control slices was determined by Student's *t*-test: $P < 0.01$ at KCl concentrations of 30 mM or greater.

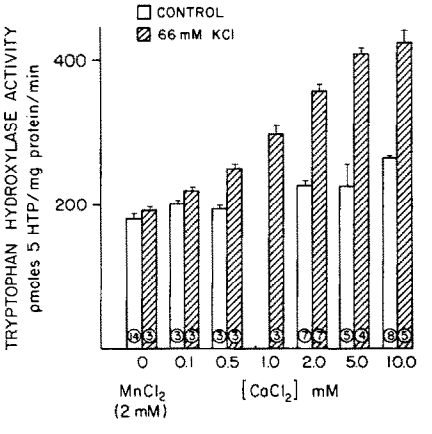

Fig. 2. Effect of increasing concentrations of $CaCl_2$ in the incubation medium on the activity of tryptophan hydroxylase from slices of rat brain stem incubated in otherwise unmodified control or 66 mM KCl media. Calcium-free medium contained 2 mM $MnCl_2$. Circled numbers indicate the number of brain stem slice preparations tested under each condition. The level of the significance of the difference between the activity of enzyme from control and depolarized tissues at a given calcium concentration was determined by Student's *t*-test. For calcium-free medium there was no significant difference. For all other calcium concentrations, except 1 mM where controls were not run, $P < 0.005$. The activity of enzyme from slices depolarized in the presence of calcium was significantly greater than that from slices depolarized in its absence (0 $CaCl_2$, 2 mM $MnCl_2$): $P < 0.001$ for all calcium concentrations except 0.1 mM, where $P < 0.01$. In the control medium, there was a significant increase in enzyme activity compared with that from calcium-free control medium at calcium concentrations of 2 mM ($P < 0.001$), 5 mM ($P < 0.02$), and 10 mM ($P < 0.001$).

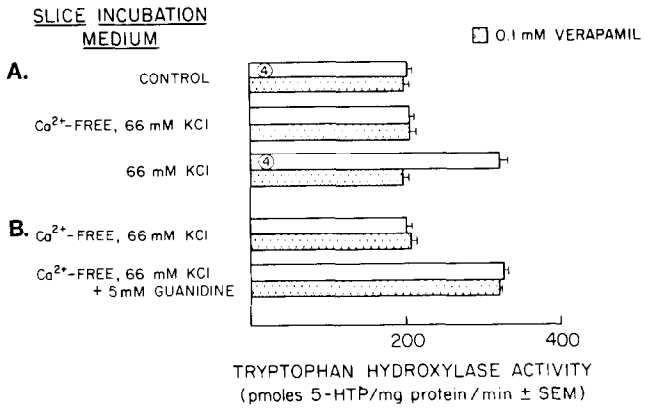

Fig. 3. Effect of verapamil on the increase in tryptophan hydroxylase activity induced by depolarization of brain stem slices in a potassium-enriched medium (A) or incubation of the slices with 5 mM guanidine (B). Brain stem slices were first rinsed for 5 min in calcium-free control medium containing 0.1 mM EGTA to rid them of extracellular calcium and were then exposed to calcium-free, potassium-enriched medium containing 0.1 mM EGTA, in the presence or absence of verapamil for 5 min. [Calcium-free control medium was also used to ensure that verapamil had no effect under these conditions (A).] In experiment A, calcium (final concentration, 2 mM) was added to the control medium and to one set of slices in potassium-enriched medium, in the presence and absence of verapamil. In experiment B, guanidine was added to the calcium-free, potassium-enriched medium. The incubation of the slices of brain stem was then continued for a further 10 min. Three brain stem slice preparations were tested under each condition unless otherwise indicated by the circled numbers.

depolarization were carried out in 66 mM KCl medium.

Effect of calcium concentration in the slice incubation medium. During depolarization, calcium crosses excitable membranes down its electrochemical gradient. By altering the extracellular calcium concentration and hence the electrochemical gradient for calcium across the membrane, it is possible to vary the intracellular concentration of ionized calcium achieved during depolarization, [19, 20]. From Figure 2 it can be seen that there was a graded increase in tryptophan hydroxylase activity as the concentration of $CaCl_2$ in the depolarizing medium was raised from 0 to 5.0 mM. No further increase in enzyme activity was observed with a calcium concentration above 10 mM in the potassium-enriched medium.

The activity of enzyme prepared from slices incubated with low (0.1 or 0.5 mM) or zero calcium in nondepolarizing control medium was 10–20% lower than that of enzyme from slices exposed to control medium containing the usual 2 mM calcium (Fig. 2) ($P < 0.005$). In contrast, raising calcium levels in control medium to 10 mM increased enzyme activity significantly over that from slices incubated in medium with 2.0 mM calcium ($P < 0.001$).

Effect of the calcium channel blocker, verapamil. The slow, voltage-dependent, tetrodotoxin-insensitive channels in membranes of excitable tissue that permit calcium ions to pass inward during depolarization [21] can be blocked in their "open" or "activated" state by verapamil [22]. An initial 5-min preincubation of the brain stem slice preparations with verapamil in calcium-free 66 mM KCl medium blocked the increase in tryptophan hydroxylase activity observed when the calcium was added to the depolarizing medium (Fig. 3A).

Is the depolarization-induced increase in tryptophan hydroxylase mediated by calcium-dependent proteases? Since tryptophan hydroxylase can be activated in supernatant preparations by calcium-dependent proteases [23], the possibility existed that

Table 1. Effects of EGTA and EDTA in the homogenizing buffer on the increase in enzyme activity induced by depolarization of slices of rat brain stem in 66 mM KCl*

Addition to homogenizing buffer	Tryptophan hydroxylase activity [pmoles 5-HTP·(mg protein)$^{-1}$·min^{-1}] Control	K-DEP	% Control
	270	417	154
4 mM EGTA	267	396	148
	280	442	158
10 mM EGTA	265	442	167
	242	364	150
20 mM EDTA	240	342	142

* For details of calculations, see Materials and Methods.

these proteases might be activated during homogenization by the raised concentration of free calcium in the depolarized slices, and that the action of these activated proteases in the disrupted tissue would then be responsible for the observed increase in tryptophan hydroxylase activity. To exclude this possibility, control and depolarized slices were homogenized in buffer containing a final concentration of 4 or 10 mM EGTA. Under these conditions, the increase in the activity of enzyme from the depolarized slices remained unchanged (Table 1). A similar outcome resulted if 20 mM EDTA was used in the homogenizing buffer.

Reversibility of the calcium-dependent, depolarization-induced activation of tryptophan hydroxylase. In this experiment, slices were incubated in either control or 66 mM KCl medium containing 2 mM $CaCl_2$ for 10 min at 25°. The slices were then transferred to fresh control medium and reincubated for various lengths of time, up to 35 min. Under these conditions, the increase in enzyme activity observed with depolarization disappeared after reincubation for 30–35 min (Fig. 4). If the slices were depolarized again after the 30-min recovery period, enzyme activity increased to 148% of control levels.

Effect of antipsychotic drugs on the depolarization-induced increase in tryptophan hydroxylase activity. The calcium-dependent activation of supernatant preparations of tryptophan hydroxylase that occurs under phosphorylating conditions has an absolute requirement for calmodulin, which was identified through the inhibitory effects of antipsychotic drugs on the activation process, the reversal of this inhibition by readdition of pure calmodulin to the enzyme, and the failure of the enzyme to become activated after removal of calmodulin by affinity chromatography [14]. In the present study, two antipsychotic drugs, haloperidol and fluphenazine, which have been demonstrated to bind to calmodulin [24, 25], were tested for their abilities to block the depolarization-induced activation of tryptophan hydroxylase. From Fig. 5 it can be seen that the activity of enzyme from depolarized slices was reduced to control levels by the addition of increasing concentrations of haloperidol or fluphenazine to the slice incubation medium. One possible explanation for this effect is that the drugs simply blocked the depolarization-induced entry of calcium into the nerve. To try to distinguish between intracellular (presumably calmodulin-mediated) and extracellular sites of action, the drugs were tested for their abilities to block the activation of tryptophan hydroxylase produced by the intracellular release of calcium ions. In these experiments, slices of rat brain stem were incubated in calcium-free control medium containing 100 μM EGTA, to which the metabolic inhibitors, rotenone or guanidine, were added. These poisons release calcium from mitochondrial stores (e.g. see Ref. 26) and activate tryptophan hydroxylase, in the

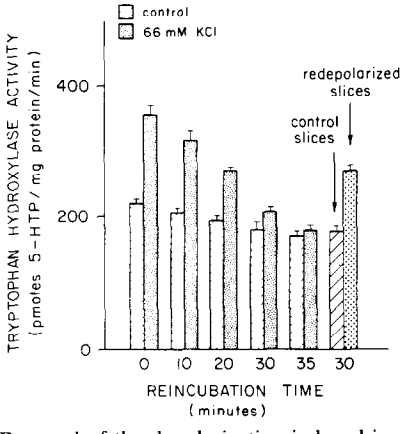

Fig. 4. Reversal of the depolarization-induced increase in tryptophan hydroxylase activity by reincubation of depolarized slices of rat brain stem in control medium. Brain stem slice preparations were incubated for 10 min at 25° in control or 66 mM KCl medium. The slices were then transferred to fresh control medium and reincubated for various lengths of time. The results are expressed as the mean ± S.E.M. of enzyme activity in pmoles 5-HTP per mg protein per min from a minimum of three different brain stem slice preparations for each condition tested. The significance of the differences between enzyme activity from control and depolarized slice preparations at different times of reincubation was determined by Student's *t*-test. At 0, 10 and 20 min, $P < 0.001$. After 30 and 35 min of reincubation, the differences were no longer significant. After 30 min of reincubation, control and depolarized slices were transferred back to fresh control (▨) or 66 mM KCl (▩) medium for 10 min. This second depolarization increased enzyme activity to 148 ± 4% of control ($P < 0.001$) compared with the initial increase of 163 ± 5%.

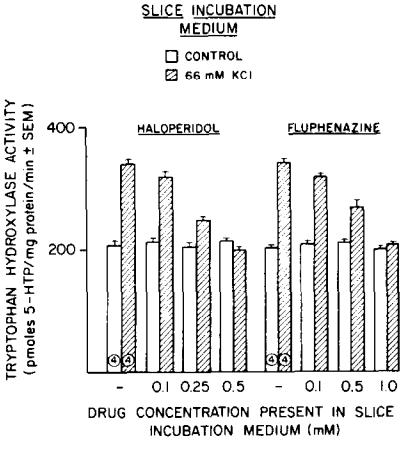

Fig. 5. Effects of haloperidol and fluphenazine on the increase in tryptophan hydroxylase activity induced by depolarization of slices of rat brain stem in a potassium-enriched incubation medium. Slice preparations were preincubated for 10 min in control media containing haloperidol or fluphenazine and were then transferred for a further 10 min of incubation to control or potassium-enriched media containing the same drug concentrations. (Haloperidol was dissolved in ethanol; the final concentration of ethanol in the medium was 5%. Controls also included ethanol.) Three brain stem preparations were usually tested under each condition, unless indicated otherwise by the circled number. The significance of the difference between the activity of enzyme from depolarized and control slices at the different drug concentrations was determined by Student's *t*-test: $P < 0.01$ for all drug concentrations except 0.5 mM haloperidol and 1.0 mM fluphenazine when differences were no longer significant.

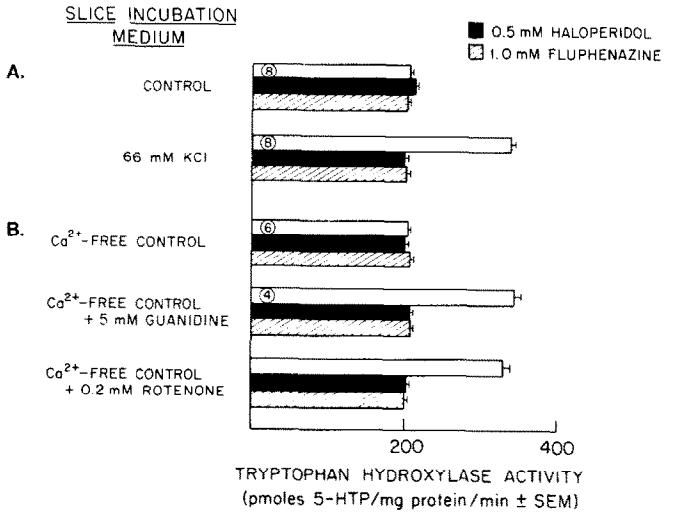

Fig. 6. Reversal by haloperidol and fluphenazine of the increase in tryptophan hydroxylase activity observed when slices of rat brain stem were depolarized in a potassium-enriched medium, or incubated in the presence of the metabolic inhibitors guanidine and rotenone. Slice preparations were preincubated for 5 min in normal control (A) or calcium-free control media (B), which were drug free or contained 0.5 mM haloperidol or 1 mM fluphenazine. Haloperidol was dissolved in ethanol which gave a final concentration of 5% ethanol in the incubation medium. All control data were pooled since ethanol had no effect on enzyme activity (see Fig. 5). In experiment A, brain stem slices were transferred to fresh control or potassium-enriched media containing no drug. (□), 0.5 mM haloperidol (■) or 1 mM fluphenazine (▩) and incubated for a further 10 min. In experiment B, guanidine or rotenone was added to the drug-free, haloperidol- or fluphenazine-treated slices, and the incubation was continued for 10 min. Three brain stem slice preparations were tested under each condition unless indicated otherwise by the circled numbers. The activity of enzyme from slices exposed to potassium-enriched medium or metabolic poisons was significantly greater than that of controls ($P < 0.001$ by the Student's *t*-test) in the absence of haloperidol or fluphenazine but did not differ from control activity in the presence of these drugs.

absence of extracellular calcium [8]. Since the kinetic properties of the enzyme obtained after such treatments are similar to those observed during potassium depolarization, the mechanisms of activation may be similar [8]. When brain stem slices were preincubated with haloperidol or fluphenazine, the effects of added guanidine or rotenone were no longer observed (Fig. 6). In contrast, verapamil had no effect on the increase in enzyme activity induced by guanidine (Fig. 3B). This was true whether verapamil was tested against guanidine in calcium-free, control medium (data not shown) or in calcium-free, depolarizing medium (66 mM KCl) which allows the verapamil to interact with the calcium channels of the nerve membrane [22] (Fig. 3B).

Effect of tetracaine on the depolarization-induced

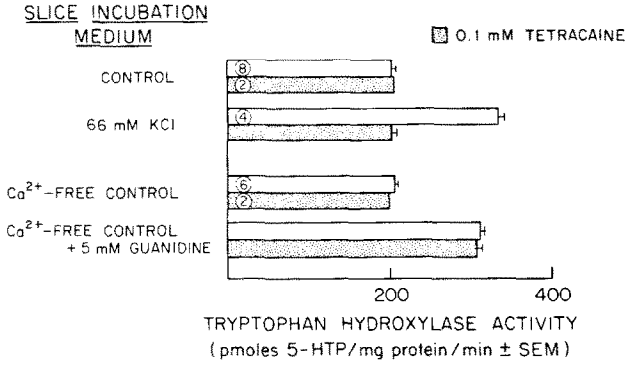

Fig. 7. Effect of tetracaine on the increase in tryptophan hydroxylase activity induced by exposure of rat brain stem slice preparations to a medium enriched in potassium or containing 5 mM guanidine. Three brain stem slice preparations were tested under each condition, unless indicated otherwise by the circled numbers. The activity of enzyme from slices depolarized in the presence of tetracaine was not significantly different from control enzyme activity, by Student's *t*-test. Activity of enzyme from guanidine-treated slices remained elevated in the presence of tetracaine ($P < 0.001$) compared with the corresponding control activity.

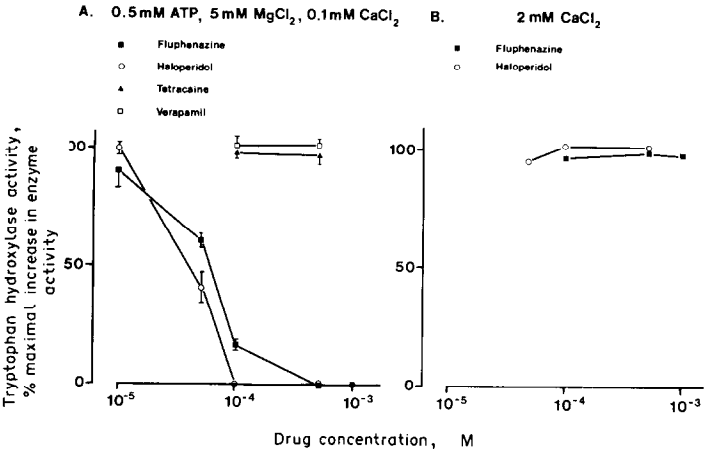

Fig. 8. Effect of drugs on the increase in tryptophan hydroxylase activity obtained when supernatant preparations of brain stem were assayed in the presence of 0.5 mM ATP, 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (A) or 2 mM $CaCl_2$ (B). The increase in enzyme activity, over that of control, observed under these conditions in the presence of various concentrations of drugs was expressed as a percentage of the increase in activity observed in the absence of drugs. Results are the mean of three different experiments (duplicate determinations) ± S.E.M. In B, the S.E.M. amounted to less than ±2%.

increase in tryptophan hydroxylase activity. The actions of haloperidol and fluphenazine were also compared with those of the local anaesthetic tetracaine [27]. Tetracaine blocked the depolarization-induced increase in enzyme activity, but it failed to inhibit the increase in enzyme activity observed when slices were incubated with 5 mM guanidine in calcium-free, control medium (Fig. 7).

Effects of drugs that block the depolarization-induced activation of tryptophan hydroxylase on the increase in enzyme activity seen under phosphorylating conditions. Verapamil and tetracaine had no effect on the increase in enzyme activity observed when supernatant preparations of brain stem were incubated in the presence of 5.0 mM $MgCl_2$, 0.5 mM ATP and 0.1 mM $CaCl_2$ (Fig. 8). In contrast, both haloperidol and fluphenazine block this increase in enzyme activity (see Ref. 14 and Fig. 8). However, neither of these antipsychotic drugs inhibited the increase in tryptophan hydroxylase activity induced by the addition of a high concentration of $CaCl_2$ to supernatant preparations of enzyme (Fig. 8).

DISCUSSION

The requirement for calcium ions in the activation of tryptophan hydroxylase induced by depolarization of brain slices in a potassium-enriched medium was identified several years ago [4, 7, 8]. What the present experiments demonstrate is that a graded increase in enzyme activity occurs in response to depolarization in the presence of increasing concentrations of extracellular calcium. This graded alteration in enzyme activity presumably reflects incremental increases in the intraneuronal concentration of ionized calcium in the vicinity of the enzyme. Direct measurements of the intracellular concentrations of free calcium achieved in response to depolarization cannot be made in a tissue preparation like brain slices. However, studies on the giant axon of the squid, using aequorin fluorescence as indicator for ionized calcium, have shown that intra-axonal ionized calcium rises with depolarization and is increased further by raising the extracellular calcium concentration or the frequency of nerve stimulation [19, 20]. These results indicate that the mechanism involved in the activation of tryptophan hydroxylase by potassium depolarization must be sensitive to the concentration of ionized calcium reached intraneuronally. Furthermore, they point to a possible explanation for the sensitivity of 5-HT synthesis to the frequency of nerve stimulation *in vivo* [2], namely the occurrence of a graded increase in the activity of the rate-limiting enzyme in 5-HT formation in response to graded increases in free intracellular calcium.

Changes in calcium concentration in control, i.e. nondepolarizing incubation medium, also have an effect on tryptophan hydroxylase activity. In this case, data from unstimulated squid axons indicated that intracellular free calcium levels rise or fall with an increase or decrease in extracellular calcium concentrations [19, 28]. Thus, the 10–20% decrease in enzyme activity observed when slices were incubated in nondepolarizing medium in the presence of low or zero calcium (2 mM $MnCl_2$) presumably reflects the effects of a lower steady-state level of free intraneuronal calcium on the mechanism that regulates enzyme activity, whereas the increase observed with 10 mM calcium resulted from the reverse situation.

The nature of the involvement of calcium ions in the depolarization-induced increased in enzyme activity has not been determined. However, *in vitro* studies on low speed supernatant preparations of tryptophan hydroxylase from rat brain stem have revealed the existence of two types of calcium-

dependent activation. The addition of high concentrations of calcium, in the millimolar range, to supernatant enzyme preparations increases tryptophan hydroxylase activity through the action of calcium-dependent proteases [23]. This mechanism of enzyme activation can be blocked by protease inhibitors such as phenylmethylsulfonylfluoride or by calcium chelation with EGTA and is, of course, irreversible. The second calcium-dependent activation is observed when the enzyme is incubated under phosphorylating conditions and is mediated by calmodulin [13, 14]. This activation requires micromolar rather than millimolar concentrations of calcium [9, 10, 12–14], and can be blocked by EGTA [9] or by antipsychotic drugs [14] which bind calmodulin [24].

Two lines of evidence now exclude any role for calcium-activated proteases in the depolarization-induced activation of tryptophan hydroxylase, either in the intact slice preparation or during homogenization of the brain stem slices. These are, respectively, the reversibility of the activation in brain stem slices reincubated in control medium and the lack of effect on the enzyme activation of high concentrations of EGTA or EDTA in the homogenizing medium. The second result also rules out activation by any other calcium-dependent process, as an artifact of homogenization.

The sensitivity of the depolarization-induced increase in tryptophan hydroxylase activity to haloperidol and fluphenazine appears consistent with a role for calmodulin in the activation of this enzyme, but one problem in interpretation of these data lies in the possibility that these drugs may simply block the depolarization-induced entry of calcium into nerve. For example, many of these drugs are known to have local anesthetic actions in concentrations above 10 μM [29], and this may be of significance since tetracaine, a long acting local anesthetic [27], will block the depolarization-induced enzyme activation just as well as the calcium-channel blocking agent, verapamil. This issue has, however, been complicated by a report that tetracaine and other local anesthetics also interact with calmodulin [30]. To analyze this problem, another approach was adopted for the activation of tryptophan hydroxylase. This involved the use of metabolic inhibitors as tools for raising the concentration of free intracellular calcium. Metabolic inhibitors have been shown to increase free calcium levels in squid axoplasm, as determined directly by aequorin fluorescence [31], to inhibit calcium uptake by mitochondria [32–34], and to enhance the spontaneous and evoked release of transmitter [26], a process triggered by a rise in intraneuronal free calcium [35]. To exclude any role for extracellular calcium, these experiments were carried out in calcium-free medium containing 100 μM EGTA. There was a striking difference in the effects of tetracaine and verapamil and the two antipsychotic drugs on the increase in enzyme activity resulting from exposure of the brain stem slices to guanidine. Neither tetracaine nor verapamil blocked the increase in enzyme activity whereas both the antipsychotic drugs did. This finding suggests strongly that the antipsychotic drugs have an intracellular rather than extracellular site of action in the brain slice and are, therefore, unlikely to inhibit the depolarization-induced increase in enzyme activity through a block of voltage-sensitive calcium channels.

In contrast, both tetracaine and verapamil seem to behave like calcium channel blockers in this system as judged by their inability to block the guanidine-induced increase in enzyme activity. In addition, they failed to inhibit the increase in enzyme activity obtained when supernatant preparations were incubated under phosphorylating conditions. Thus, neither drug appears to interact with calmodulin (at least not in a way that alters tryptophan hydroxylase activity) or with any of the other components reported to be involved in calcium–calmodulin-dependent enzyme activation under phosphorylating conditions, namely calcium–calmodulin-dependent protein kinase [15, 36] and a 70,000 dalton activator protein that is distinct from calmodulin [36].

The known interactions of antipsychotic drugs with calmodulin [24, 25] make it likely that the depolarization-induced activation of tryptophan hydroxylase involves a calmodulin-regulated process. This view is also supported by similarities between depolarization-induced enzyme activation and the calmodulin-dependent activation obtained with cell-free enzyme preparations under phosphorylating conditions, e.g. reversibility, calcium requirement and sensitivity to both calcium concentration and antipsychotic drugs. It should be noted, however, that these properties are also common to other calcium- or calmodulin-sensitive regulatory enzymes, namely, calmodulin-sensitive adenylate cyclase [37] and calcium-activated, phospholipid-dependent protein kinase [38–41]. Thus, it becomes necessary to consider whether these enzymes participate in the regulation of tryptophan hydroxylase activity by depolarization.

Both crude supernatant and partially purified preparations of tryptophan hydroxylase are activated by phospholipids, but this occurs without the presence of ATP or magnesium [42] and, thus, suggests that a direct interaction with tryptophan hydroxylase may be involved. The question as to whether phospholipid-dependent protein kinase activates the enzyme in cell-free preparations has not been examined but seems unlikely since phospholipid-dependent protein kinase activity is inhibited by tetracaine and verapamil in concentrations that leave the activation of tryptophan hydroxylase produced under phosphorylating conditions totally unaffected (Ref. 40 and Fig. 8). These observations however, do not address the question of whether tryptophan hydroxylase may be activated by a phospholipid-dependent protein kinase in intact neurones, when the phospholipid environment of the enzyme may very well be quite different from that of the cell-free enzyme extract.

The question whether cyclic AMP plays a role in the regulation of tryptophan hydroxylase activity and could possibly act as a mediator of the depolarization-induced enzyme activation via activation of, say, calmodulin-dependent adenylate cyclase was re-examined recently [43]. Dibutyryl cyclic AMP increases 5-HT synthesis *in vivo* after intracerebroventricular administration and activates

tryptophan hydroxylase after preincubation in slice preparations [44, 45]. However, 8-bromo cyclic AMP does not increase tryptophan hydroxylase activity if included in the slice incubation medium, and several other treatments of the brain stem slices, such as exposure to cholera toxin, adenosine or 2-chloroadenosine which would be expected to raise intraneuronal levels of cyclic AMP [46, 47], likewise have no effect on tryptophan hydroxylase activity [43]. Phosphodiesterase inhibitors of the xanthine type do increase enzyme activity [43], but only in such high (mM) concentrations that they undoubtedly produce many other effects besides an increase in cyclic AMP levels [48]. These concentrations of xanthines mobilize calcium from intracellular storage sites, such as the sarcoplasmic reticulum of muscle [49], a calcium-sequestering organelle that has its counterpart in nerve [50–52]. The fact that the effects of caffeine, as well as of dibutyryl cyclic AMP, are blocked by haloperidol and fluophenazine [43] suggests that calmodulin-dependent protein kinase, rather than cyclic AMP or some other nucleotide, mediates the enzyme activation. The failure of papaverine, a phosphodiesterase inhibitor structurally unrelated to the xanthines, to alter tryptophan hydroxylase activity, even when tested on the slices of brain stem in millimolar concentrations, is also consistent with this interpretation, as is the inability of cyclic AMP or any other cyclic nucleotides to increase tryptophan hydroxylase activity in low speed supernatant preparations of brain stem incubated in the presence or absence of ATP and magnesium [9]. Thus little support exists for cyclic AMP as a regulator of tryptophan hydroxylase.

On balance, the inhibitory effects of haloperidol and fluphenazine on the depolarization-induced activation of tryptophan hydroxylase, taken together with the evidence for activation of the enzyme in supernatant preparations by a calcium–calmodulin-dependent protein kinase, suggests that calmodulin-sensitive protein kinase mediates the calcium-dependent activation induced by depolarization.

One important question raised by the present observations is the relation the depolarization-induced activation of tryptophan hydroxylase bears to the frequency-dependent increase in 5-HT synthesis that occurs *in vivo* in response to nerve stimulation. *In vitro* studies indicate that the increase in 5-HT synthesis seen in response to depolarization is calcium dependent [6] and cannot be accounted for by increases in tissue tryptophan concentrations [5]. On the other hand, although activation of crude preparations of enzyme can be readily demonstrated as a result of depolarization of brain slices, such an activation has not been convincingly demonstrated following *in vivo* nerve stimulation [53]. Bourgoin and her coworkers failed to obtain an increase in enzyme activity even under conditions where 5-HT synthesis was elevated 50% by electrical stimulation and continued at a level well above that of unstimulated control tissue for 30 min after stimulation stopped. This failure to demonstrate increased enzyme activity *in vitro* after *in vivo* stimulation may indicate that the 5-HT neuron has other mechanisms besides activation of tryptophan hydroxylase for accelerating 5-HT synthesis during neuronal activity. On the other hand, it is also conceivable that in experiments in which neurons are selectively depolarized by electrical stimulation tryptophan hydroxylase becomes rapidly deactivated once the tissue has been homogenized. Although at present there is no evidence that the depolarization-induced activation involves enzyme phosphorylation, it is instructive to consider this model. Once an enzyme has undergone phosphorylation, then for it to stay phosphorylated, the activity of the specific protein kinase mediating its phosphorylation must remain higher than that of the phosphoprotein phosphatase which dephosphorylates it. Dephosphorylation presumably could occur during homogenization of tissues even at ice-cold temperatures. However, the activity of enzyme from depolarized slices is only increased by about 10% when the phosphoprotein phosphatase inhibitor, NaF, is included in the homogenizing medium in a concentration of 12 mM (M. Boadle-Biber, unpublished data). Presumably it will be possible to resolve these discrepancies using a more sensitive assay procedure for tryptophan hydroxylase after *in vivo* nerve stimulation with precautions taken to prevent *in vitro* phosphorylation or dephosphorylation of protein. The widespread occurrence of protein phosphorylation as a mechanism for regulating enzyme activity [54–56] including, it seems, that of tryptophan hydroxylase [15, 36] provides a possible role for protein phosphorylation in the control of tryptophan hydroxylase activity in response to potassium depolarization of slices *in vitro* and nerve activity *in vivo* worthy of vigorous investigation.

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