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Blockade by haloperidol of the increase in tryptophan hydroxylase activity induced by incubation of slices of brain stem with dibutyryl cyclic AMP

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A discrepancy exists in the literature concerning the role of cyclic AMP in the regulation of the activity of tryptophan hydroxylase [tryptophan-5-monooxygenase, L-tryptophan, tetrahydropterin: oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4] from rat brain. Low speed supernatant preparations of this enzyme are reversibly activated under phosphorylating conditions by a process that is enhanced by the addition of micromolar amounts of calcium and requires the presence of calmodulin, but which is unaffected by cyclic AMP or any other cyclic nucleotides [1–5]. On the other hand, the *in vivo* conversion of tryptophan to 5-HTP* in the presence of an aromatic amino acid decarboxylase inhibitor is increased markedly following administration of dibutyryl cyclic AMP via the intracerebroventricular route [6]. This action of dibutyryl cyclic AMP cannot be explained by changes in tryptophan availability and may, therefore, arise from an activation of tryptophan hydroxylase [6]. Consistent with this suggestion is the finding that incubation of slices of rat brain stem with dibutyryl cyclic AMP produces an activation of tryptophan hydroxylase which can be demonstrated by kinetic measurements made on low speed supernatant fractions of the enzyme prepared from the pretreated slices [7]. The occurrence of this activation is surprising in view of the failure of cyclic AMP to increase tryptophan hydroxylase activity when added to low speed supernatant fractions in the presence or absence of phosphorylating conditions. To determine whether endogenous cyclic AMP has any action on tryptophan hydroxylase, a study was made in which slices of rat brain stem were exposed to substances which increase endogenous cyclic AMP levels in nervous tissue (phosphodiesterase inhibitors [8], cholera toxin [9], adenosine and 2-chloroadenosine [10]). In addition, another cyclic AMP analogue, 8-bromo cyclic AMP, and the antipsychotic drug,

* Abbreviations: 5-HTP, 5-hydroxytryptophan; $6MPH_4$, DL-6-methyl-5,6,7,8-tetrahydropterin; brocresine (NSD 1055), 3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate; dibutyryl cyclic AMP, N^6,$O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate; 8-bromo cyclic AMP, 8-bromoadenosine 3′:5′-cyclic monophosphate; and EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*′tetraacetic acid.

haloperidol, which blocks calmodulin-mediated reactions [11], were tested for effects on enzyme activity following addition to the brain stem slice preparation.

Materials and methods

Groups of male Sprague–Dawley rats of matched weights (150–175 g) were used in this study. Each enzyme preparation was obtained from a single rat brain stem which had been cut into 250 μm slices with a Dupont Sorvall MT-2 tissue chopper and incubated for 10 min at 25° in 5 ml of oxygenated medium [150 mM, NaCl; 6 mM, KCl; 1 mM, $MgCl_2$; 2 mM, $CaCl_2$; 10 mM, glucose; 10 mM, Tris acetate buffer (pH 7.4)], to which additions had been made as indicated in Results and Discussion. At the end of the incubation, the slices were separated from the medium by gentle centrifugation (10,000 *g* for 10 min) at 4° in a 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 centrifuged at 39,000 *g* for 30 min to obtain a low speed supernatant fraction. To this, dithiothreitol was added in a final concentration of 2 mM, and the pink-colored enzyme supernatant fraction was then passed over a Sephadex G-25 column (22 × 1.3 cm) equilibrated with 0.05 M Tris acetate (pH 7.4) containing 2 mM dithioreitol at 7°, to remove endogenous tryptophan and other indoles which raise the blank fluorescence of the enzyme assay. The tryptophan hydroxylase assay was a modification of that of Friedman *et al.* [12] in which the formation of 5-HTP from tryptophan, in the presence of the aromatic amino acid decarboxylase inhibitor brocresine (NSD 1055, 3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate) and an artificial reduced pterin cofactor, $6MPH_4$, is measured by its fluorescence in HCl (excitation, 305 nm; emission, 535 nm). Details of the procedures have been given in two earlier publications [13, 14]. Enzyme activity was assayed in the presence of subsaturating concentrations of $6MPH_4$ (50 μM) and barely saturating concentrations of substrate, L-tryptophan (200 μM), to screen for potential activation of the enzyme. Assays were carried out in quintuplet or sextuplet and were always checked for linearity with time and protein. Protein was determined by the procedure of Lowry *et al.* [15] using bovine serum albumin as standard. Results are expressed as per cent of control activity or in pmoles 5-HTP per mg protein per min ± standard error of the mean.

The sources of the various drugs and chemicals used in this study were as follows: dibutyryl cyclic AMP (sodium salt), 8-bromo cyclic AMP (sodium salt), papaverine HCl, caffeine, theophylline, 3-isobutyl-1-methylxanthine and 2-chloroadenosine from the Sigma Chemical Co., St. Louis, MO; adenosine, cholera toxin and $6MPH_4$ from the Calbiochem–Behring Corp., La Jolla, CA; haloperidol from McNeil Pharmaceutical, Spring House, PA; brocresine from Lederle Laboratories, Pearl River, NY; and verapamil from the Knoll Pharmaceutical Co., Whippany, NJ. Tetracaine (Mann Research Laboratories, NY) was a gift of Dr. Ronald Rubin, Department of Pharmacology, Medical College of Virginia.

Results and discussion

Figure 1 summarizes the effects on tryptophan hydroxylase activity of pretreatment of slices of rat brain stem with dibutyryl cyclic AMP and four phosphodiesterase inhibitors. There was a maximum increase in enzyme activity of 70% if the slices were incubated with 2.5 mM dibutyryl cyclic AMP, as reported previously [7], and this was not changed significantly if the calcium in the incubation medium was replaced with manganese. Direct addition of this same concentration of dibutyryl cyclic AMP to the enzyme assay medium had no effect on enzyme activity (data not shown). Increases in enzyme activity of similar magnitude were obtained when the slices of brain stem were exposed to phosphodiesterase inhibitors of the xanthine class, namely 3-isobutyl-1-methylxanthine, and caffeine; theophylline had significant but less marked effects. Incubation of the slices with these inhibitors increased the activity of tryptophan hydroxylase when it was assayed in the low speed supernatant fraction but had no effects when added in the same concentrations to the enzyme assay (data not shown). The increase in enzyme activity was also observed when extracellular calcium was replaced with manganese, though it was not quite as large with caffeine. It should be noted that the concentrations of these inhibitors required to produce the increase in tryptophan hydroxylase activity were very high indeed, a situation which raises serious concerns about the selectivity of their action, especially since, at these concentrations, caffeine and theophylline are known to interfere with the uptake of calcium by the sarcoplasmic reticulum of muscle [16], a calcium-sequestering organelle which has its counterpart in nerve [17]. The additional finding that high concentrations of the structurally unrelated phosphodiesterase inhibitor, papaverine, were virtually without effect on tryptophan hydroxylase activity, when included in the slice incubation medium (22% increase at 40 mM) (Fig. 1), also indicates that the actions of the xanthines on tryptophan hydroxylase activity are unlikely to be related to their inhibition of phosphodiesterase and the associated accumulation of endogenous cyclic AMP. Three other substances, cholera toxin (1 μg/ml, 15 and 30 min), adenosine and 2-chloroadenosine (10^{-5} and 10^{-4} M, 10 min), which are also known to raise endogenous cyclic AMP levels [9, 10], likewise failed to alter tryptophan hydroxylase activity after incubation with the brain stem slice preparation. These findings thus appear to exclude any role for endogenous cyclic AMP in modulating the kinetic behavior of tryptophan hydroxylase, at least in ways that remain detectable *in vitro*. This conclusion is strengthened by the failure of 8-bromo cyclic AMP to produce any change in enzyme activity when included in the slice incubation medium in concentrations of 1 and 2.5 mM. Nevertheless, the question remains as to how dibutyryl cyclic AMP and the xanthine derivatives enhance the activity of tryptophan hydroxylase. Butyric acid, a possible contaminant in preparations of dibutyryl cyclic AMP, had no action on enzyme activity when included in the slice incubation medium in concentrations of 1, 2.5 and 5.0 mM. One possibility is that the xanthines and dibutyryl cyclic AMP are all able to mobilize intracellular calcium. Tryptophan hydroxylase is readily activated by treatments to the brain stem slices which raise the intraneuronal concentration of ionized calcium. This can be achieved by exposure of the slices of brain stem to agents which promote the entry of extracellular calcium, but which are without effect on enzyme activity when the slices are incubated in calcium-free medium (e.g. depolarizing medium, ionophore A23187, ouabain [14]) or to agents, like metabolic poisons, which interfere with the intracellular storage of calcium by mitochondria and are generally effective in a calcium-free incubation medium [14].

The depolarization-induced activation of tryptophan hydroxylase is blocked by the addition of haloperidol to the slice incubation medium [18]. The haloperidol does not appear to act as a local anesthetic or calcium channel blocker for it also prevents the activation of tryptophan hydroxylase induced by metabolic poisons in a calcium-free medium [18], whereas tetracaine, a local anesthetic [19], and verapamil, a calcium channel blocker [20], do not (M. C. Boadle-Biber, in press). It is therefore likely that the haloperidol has an intracellular site of action and that the depolarization-induced activation of the enzyme is a calmodulin-mediated event as in the case of the calcium-dependent activation of supernatant preparations of enzyme incubated under phosphorylating conditions [5]. In view of these recent observations, haloperidol was tested to see if it would block the increase in tryptophan hydroxylase activity induced when brain stem slices are incubated

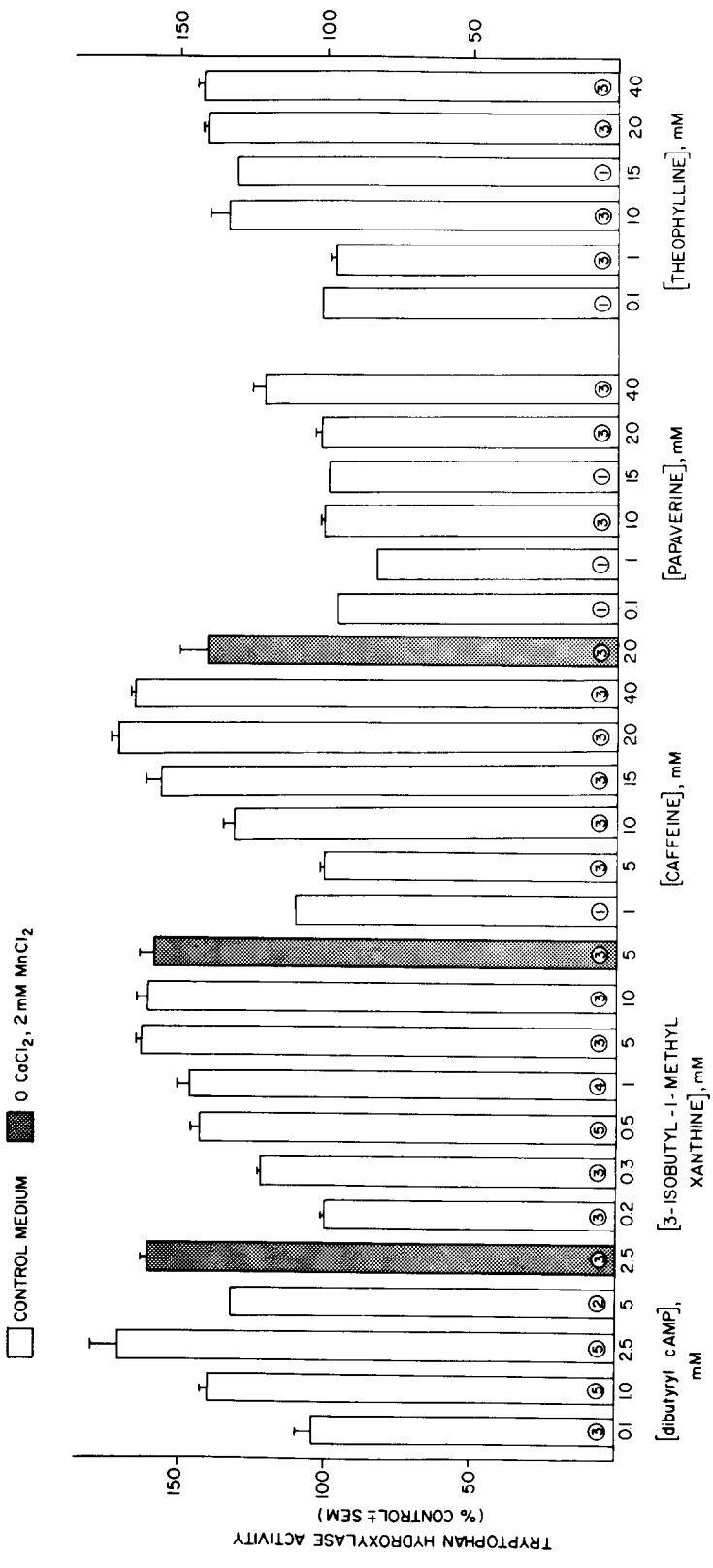

Fig. 1. Effects of dibutyryl cyclic AMP and various phosphodiesterase inhibitors on the activity of tryptophan hydroxylase prepared from pretreated slices of rat brain stem. Circled numbers indicate N, the number of brain stem slice preparations tested under each condition. Significance of the difference between enzyme activity from treated and control slices was determined by Student's *t*-test for values of N of three or more: $P < 0.05$ for all drug concentrations except 0.1 mM dibutyryl cyclic AMP, 0.2 mM 3-isobutyl-1-methylxanthine, 5 mM caffeine, 10 and 20 mM papaverine, and 1 mM theophylline when differences were not significant.

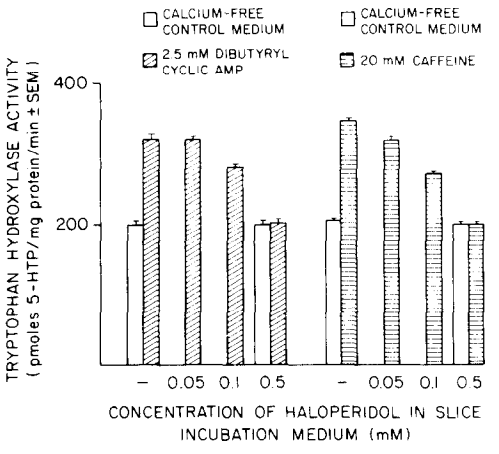

Fig. 2. Effect of increasing concentrations of haloperidol on the increase in tryptophan hydroxylase activity induced by the presence of dibutyryl cyclic AMP or caffeine in the slice incubation medium. Haloperidol was dissolved in absolute ethanol; the final concentration of ethanol in the experimental and control media was 5%. Brain stem slice preparations were preincubated with haloperidol in calcium-free medium containing 100 μM EGTA for 10 min prior to the addition of dibutyryl cyclic AMP or caffeine. A minimum of three brain stem slice preparations was tested under each condition.

in the presence of dibutyryl cyclic AMP or caffeine. Figure 2 illustrates that increasing concentrations of haloperidol, in a calcium-free medium, reduced and then abolished the effect of 2.5 mM dibutyryl cyclic AMP and 20 mM caffeine on tryptophan hydroxylase activity. The drug was without effect on enzyme prepared from slices incubated only in calcium-free control medium. The concentration of haloperidol which produced a complete block of the increase in enzyme activity was 500 μM, only five times that necessary to block the activation of the enzyme in cell-free systems, when low speed supernatant preparations are incubated under phosphorylating conditions [5].

This ability of haloperidol to block the increase in tryptophan hydroxylase activity induced by dibutyryl cyclic AMP and the methylxanthines is not apparently shared by the local anesthetic, tetracaine, or the calcium channel blocking agent, verapamil, both drugs that exert their effects through interactions at the cell membrane. For instance, neither tetracaine nor verapamil, in concentrations of 100 μM, blocked the increase in enzyme activity that resulted when brain stem slices were incubated with 20 mM caffeine or 1 mM 3-isobutyl-1-methylxanthine respectively (Fig. 3). Yet the same concentration of these drugs blocked the depolarization-induced increase in enzyme activity (Fig. 3). This is in marked contrast to the situation with haloperidol which blocks the increase in enzyme activity produced by depolarization [18], metabolic inhibitors [18], dibutyryl cyclic AMP and the methylxanthine, caffeine, at the same concentration of 500 μM. Thus, haloperidol presumably has a site of action different from that of tetracaine and verapamil. Similar observations have also been made with another antipsychotic drug, fluphenazine (data not shown).

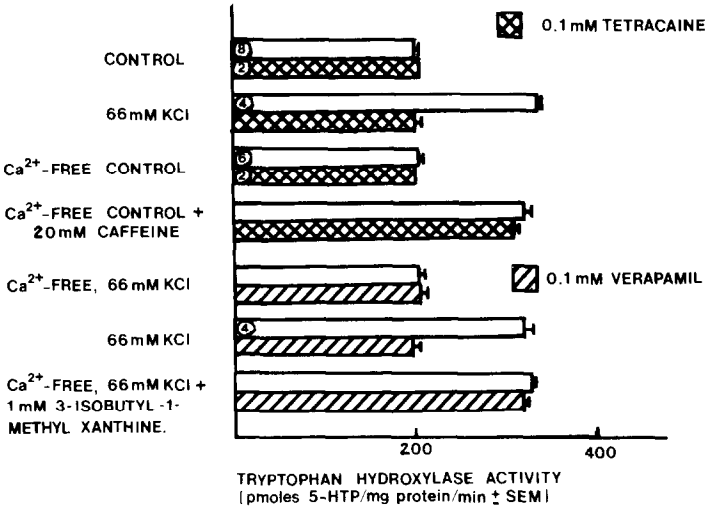

Fig. 3. Effects of tetracaine and verapamil on the increase in tryptophan hydroxylase activity induced by depolarization of brain stem slices or exposure of the slices to the methylxanthines, caffeine or 3-isobutyl-1-methylxanthine, in calcium-free medium. The depolarizing medium contained 66 mM KCl and 90 mM NaCl; other constituents were unchanged. All calcium-free media contained 100 μM EGTA. In the experiments with tetracaine, the brain stem slice preparations were exposed to control, depolarizing or caffeine-containing medium in the presence or absence of 100 μM tetracaine for 10 min. In the experiments with verapamil, brain stem slice preparations were first rinsed for 5 min in calcium-free control medium to rid them of extracellular calcium, and were then exposed to calcium-free depolarizing medium in the presence or absence of verapamil for 5 min to allow the verapamil to interact with the "open" or "activated" voltage sensitive calcium channels [20, 21]. Calcium (2 mM $CaCl_2$) was then added back to one set of slices and 1 mM 3-isobutyl-1-methylxanthine to another in the presence or absence of verapamil. Control slices were incubated in calcium-free depolarizing medium in the presence or absence of verapamil. 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.

Although this evidence is consistent with the involvement of calmodulin in the increase in enzyme activity induced by dibutyryl cyclic AMP and the methylxanthines, it is also possible that other, as yet undefined, interactions may contribute to the action of haloperidol in the brain stem slice preparation. What does emerge from these experiments is strong evidence against the involvement of cyclic AMP or any other endogenous cyclic nucleotide in the regulation of tryptophan hydroxylase in the slice preparation.

In summary, pretreatment of rat brain stem slices with dibutyryl cyclic AMP, caffeine, theophylline and 3-isobutyl-1-methylxanthine increased the activity of tryptophan hydroxylase in supernatant preparations of enzyme made from the slices. This effect does not appear to be mediated by a cyclic AMP sensitive mechanism since it was not reproduced by exposure of the slices to 8-bromo cyclic AMP, to papaverine, a nonxanthine phosphodiesterase inhibitor, or to other treatments known to raise tissue cyclic AMP levels. The ability of haloperidol to block this increase in enzyme activity is consistent with a role for calmodulin and calcium as mediators of the enzyme activation, particularly when this observation is considered in conjunction with the evidence that supernatant preparations of this enzyme are activated under phosphorylating conditions by a calcium–calmodulin dependent process [5]. Nevertheless, in view of the high concentrations of haloperidol employed in the present experiments, the possibility that this drug may produce its effects in the brain stem slices through some other action, unrelated to its ability to bind to calmodulin, should be kept in mind.

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Increased sensitivity of the fluorometric method of Snyder and Hendley for oxidase assays

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Snyder and Hendley [1] and Guilbault *et al.* [2] developed a fluorometric assay for amine oxidases, in which hydrogen peroxide formed in the oxidase reaction is measured fluorometrically by converting homovanillic acid to a highly fluorescent compound in the presence of peroxidase. This method is widely used because of its simplicity and high sensitivity, and it is applicable to any hydrogen peroxide-generating system. We have found that much higher sensitivity of this method can be achieved by changing the final pH of reaction mixtures, the details of which are reported in this communication.

As a monoamine oxidase preparation, crude mitochondrial fractions were isolated from the pooled livers of six male Sprague–Dawley rats as described previously [3]. Purified hog kidney diamine oxidase was purchased from the Sigma Chemical Co., St. Louis, MO.

The incubation mixtures contained 0.10 ml of 0.25 M sodium phosphate buffer (pH 7.4), 0.10 ml of peroxidase solution (0.5 mg/ml), 0.10 ml of homovanillic acid solution (1.0 mg/ml), 0.10 ml of enzyme solution (rat liver monoamine oxidase, 0.0159 mg protein/ml; hod kidney diamine oxidase, 0.20 mg/ml; or water for the hydrogen peroxide assay), 0.10 ml of substrate solution (benzylamine for monoamine oxidase, final concentration 1.0 mM; putrescine for diamine oxidase, final concentration 1.0 mM; or hydrogen peroxide, 22 nmoles/ml) and 0.10 ml of water.