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Tetrodotoxin inhibition *in vitro* of protoveratrine A-activated glutamate decarboxylase in synaptosomes

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Since Gray and Whittaker [1] characterized the morphology of a crude mitochondrial fraction (P_2) and found that it contained nerve-ending particles (synaptosomes), this subcellular fraction has been used to study many neurochemical phenomena, including: uptake of precursors and synthesis of neurotransmitter substances [2–6], potassium-stimulated release of dopamine [5] and norepinephrine [7], accumulation of ^{45}Ca following depolarization [8], calcium-dependent depolarization-stimulated release of monoamines [9] including gamma-aminobutyric acid (GABA) [10, 11], and high-affinity, stereospecific uptake of catecholamines [12]. This synaptosomal preparation (crude mitochondrial fraction, P_2) provides an easily interpretable and reliable system for pharmacological manipulation of these variables and demonstration of effects *in vitro* of compounds known to act at the nerve terminal [13–16].

GABA release [17–19] and increased glutamic acid decarboxylase(L-glutamic acid-1-carboxylyase; EC 4.1.1.1.5; GAD) activity [20–21] have been demonstrated following depolarization of brain slices. Although both neurotransmitter release and increased activity of the rate-limiting step for neurotransmitter biosynthesis have been observed following depolarization, the trigger mechanism for the increase in neurotransmitter synthesis has yet to be defined for the GABAergic systems. The synaptosomal preparation may prove to be a valuable tool for investigating the link between these electrophysiological and neurochemical changes. The activation of GAD in striatal slices [20, 21] by depolarizing stimuli has prompted us to investigate further the mechanism of this change using striatal synaptosomes.

L-[1-^{14}C]Glutamate was purchased from the New England Nuclear Corp. (Boston, MA). Methyl benzethonium hydroxide (Hyamine), protoveratrine A (PVA), and tetrodotoxin (TTX) were obtained from the Sigma Chemical Co. (St. Louis, MO).

Male Sprague–Dawley-derived rats, 150–350 g (Charles River Breeding Laboratories, Wilmington, MA), were housed four to a cage, maintained on a 12-h diurnal cycle, and allowed free access to food and water. The rats were decapitated and the brains were removed and rinsed in ice-cold saline. Corpora striata were dissected free, pooled in ice-cold, oxygenated 0.32 M sucrose (1 pair of striata/ml of sucrose), and homogenized in Potter–Elvehjem vessels (Kontes). The nuclei and cellular debris were separated by centrifugation at 1000 *g* for 10 min at 0–4°. The supernatant fraction was decanted into fresh centrifuge tubes and spun at 17,000 *g* for 15 min at 0–4°. The resulting pellet (P_2), containing nerve-ending particles, was resuspended in the original volume of normal Krebs–Ringer buffer (KRI) with the following composition: 122 mM NaCl, 1.3 mM $CaCl_2$, 4.9 mM KCl, 1.2 mM $MgSO_4$, 11.1 mM dextrose, and 15.9 mM imidazole-acetic acid buffer, pH 7.4. KRI containing protoveratrine A, 49 mM KCl, and TTX was substituted when necessary. The P_2 fraction was preincubated at 37° for 10 min and centrifuged at 17,000 *g* for 15 min; the supernatant fraction was decanted, and the pellet was resuspended in an equal volume of 0.32 M sucrose.

The enzyme reaction was started by adding 0.1 ml of the pretreated synaptosome suspension to Corex tubes containing KRI and 300 μM L-[1-^{14}C]glutamate (sp. act. = 6.67 mCi/mmole). This concentration was used to eliminate glutamate transport as a rate-limiting factor, because half-maximal transport of glutamate by high-affinity systems in rat CNS occurs below 20 μM [22]. The tubes were capped with serum stoppers, and $^{14}CO_2$ was trapped on 1 × 2 cm filter paper strips that were saturated with 35 μl Hyamine and suspended from hooks in the stoppers. The reaction was terminated after 10 min by the injection of 0.5 ml of 3 N H_2SO_4. Blank values were obtained by incubating the buffer containing the radiolabeled substrate in the absence of tissue. Protein concentrations were determined by the method of Lowry *et al.* Statistical evaluation of the data was carried out by one-way analysis of variance followed by the Newman–Keuls multiple comparison test.

To establish continuity with existing data [20, 21], synaptosomes prepared from corpus striatum were exposed to depolarizing stimuli during preincubation and then assayed for GAD activity. Exposure of synaptosomes to 49 mM K^+ or 100 μM PVA increased GAD activity by 21 and 28 per cent, respectively (Fig. 1). This finding is consistent with those earlier reports.

A graded increase in GAD activity, directly related to the time of exposure of striatal slices to a depolarizing concentration of K^+, has been observed [21]. In the present study, PVA (Fig. 2), a veratrum alkaloid that depolarizes by opening membrane Na^+ channels [24], and K^+ (Fig. 3) produced concentration-related increases in GAD activity when incubated with striatal synaptosomes. The increase in enzyme activity due to PVA was maximal at a concentration of 100 μM; this response was completely blocked by 5 μM TTX, a compound that blocks Na^+ channels [25]. Conversely, maximal stimulation was not observed with the concentrations of K^+ used nor was the increase due to K^+ blocked by TTX.

In many neuronal systems, increased impulse flow or electrochemical stimulation has been correlated with

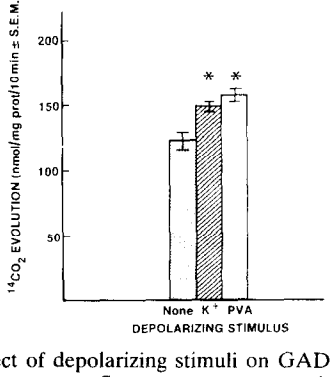

Fig. 1. Effect of depolarizing stimuli on GAD activity in striatal synaptosomes. Synaptosomes were preincubated in the presence of normal Krebs–Ringer buffer, elevated potassium (49 mM)–Krebs–Ringer buffer, or Krebs–Ringer buffer containing 100 μM protoveratrine A (PVA), for 10 min at 37°. Enzyme activity was determined with incubation in normal Krebs–Ringer buffer. Data are the means of four incubations per group ± S.E.M. An asterisk(*) denotes $P < 0.05$.

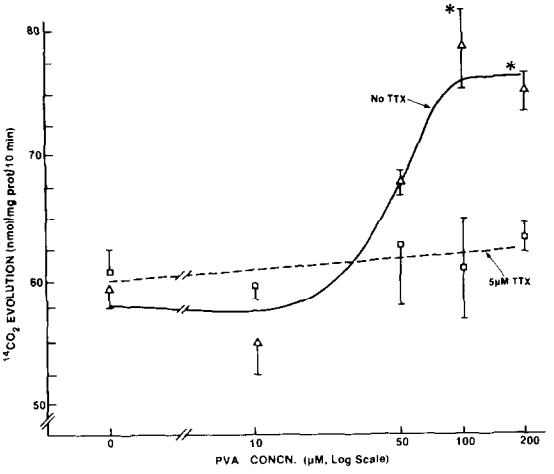

Fig. 2. Effect of PVA concentration on GAD activity in striatal synaptosomes in the presence and absence of TTX. Synaptosomes were preincubated in normal Krebs–Ringer buffer containing the indicated concentrations of PVA or TTX for 10 min at 37°. Enzyme activity was determined in the presence of normal Krebs–Ringer buffer. Data are the means of four incubations per group ± S.E.M. An asterisk (*) denotes $P < 0.05$.

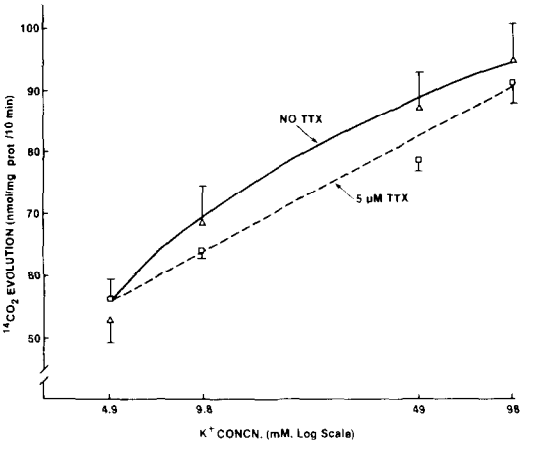

Fig. 3. Effect of potassium concentration on GAD activity in striatal synaptosomes in the presence and absence of TTX. Synaptosomes were preincubated in Krebs–Ringer buffer containing the indicated concentrations of potassium or TTX for 10 min at 37°. Enzyme activity was determined in the presence of normal Krebs–Ringer buffer. Data are the means of eight incubations per group ± S.E.M.

increased activity of the rate-limiting step for neurotransmitter biosynthesis [3, 4, 20, 21,26–28]. It is widely accepted that tyrosine hydroxylase activity is modulated by feed back inhibition by norepinephrine and, therefore, its increased activity after neuronal depolarization may be coupled to release and depletion of critical intraneuronal stores of the neurotransmitter. The role of release phenomena in the regulation of GABA is unclear at this time.

Release of GABA by K^+-enriched media is a Ca^{2+}-dependent phenomenon [11, 19, 29], but there are conflicting reports concerning Ca^{2+} dependency of release induced by congeners of veratridine [11]. Redburn *et al.* [11] have reported Ca^{2+}-dependent GABA release by veratridine. Neal [19], however, has seen a potentiation of veratridine release in the absence of extracellular Ca^{2+}.

Synaptosomal uptake of glutamate, the amino acid precursor of GABA, is increased following K^+-depolarization [30] but this increase is not dependent on calcium concentration, leading those authors to hypothesize that post-depolarization activation of glutamate uptake is not due to depletion of intraneuronal GABA stores. Interestingly, Blaustein [8] observed that TTX blocks veratridine- but not K^+-induced accumulation of ^{45}Ca by synaptosomes, suggesting a voltage-dependent mechanism for the increase in Ca^{2+} permeability.

Our data also suggest to us that a voltage-dependent increase in GAD activity follows the depolarization of striatal synaptosomes. This could be due to changes in intracellular Ca^{2+} distribution, in enzyme distribution, to an interaction of the two [31, 32] or to some other intracellular second messenger.

In summary, K^+ and PVA increased GAD activity in striatal synaptosomes. Activation by depolarizing stimuli was related to the magnitude of the stimulus. PVA activation was sensitive to TTX but K^+ activation was not.

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