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INCREASE IN SYNAPTOSOMAL GLUTAMATE DECARBOXYLASE ACTIVITY UNDER THE INFLUENCE TETANUS TOXIN

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Tetanus toxin (TT) disturbs synaptic secretion of GABA [11]. Investigation of the action of TT on isolated nerve endings (synaptosomes) revealed a decrease in the transmembrane K^+ gradient [6, 3] and membrane depolarization [16], and disturbance of the release and uptake of mediators [2, 4, 6, 10]. The decrease in synaptic secretion of GABA may be explained by inhibition of glutamate decarboxylase (GDC) (EC 4.1.1.15), which synthesizes GABA. Meanwhile the membrane-mediated increase in GABA synthesis may be an indication of depolarization of the nerve ending membrane. The study of GDC activity in synaptosomes is therefore interesting as a means of elucidating the target and mechanism of action of TT.

Accordingly in the investigation described below the effect of TT was studied directly on GDC and also on the glutamate-decarboxylase activity of nerve endings during poisoning with TT *in vivo* and *in vitro*.

EXPERIMENTAL METHOD

TT purified by gel filtration [8] was used. To obtain local tetanus, 0.2 MLD (for rats) TT in 0.5 ml physiological saline was injected into the gastrocnemius muscles of both hind limbs of albino rats weighing 180-220 g. The lumbar enlargement of the spinal cord was removed 48 h later from seven to 10 rats treated with TT and the same number of healthy animals. Spinal synaptosomes were isolated and GDC activity determined in the experimental and control groups parallel in each experiment. Spinal cord tissue was homogenized in 10 volumes 0.32 M sucrose in a glass homogenizer with Teflon pestle. The suspension was centrifuged on the TsLR-1 centrifuge (1000g, 10 min, at 2°C) and the residue was resuspended and centrifuged under the same conditions. The two supernatants were pooled and centrifuged at 15,000g for

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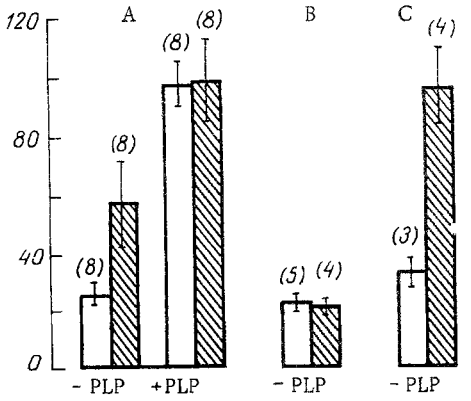

Fig. 1. Effect of tetanus toxin on GDC activity of an unpurified fraction of spinal "synaptosomes." Shaded columns — GDC activity in rats with local tetanus, unshaded columns — the same in healthy animals. A) Averaged data of all experiments; B, C) results of experiments carried out in spring and the fall respectively. —PLP) Basic activity; +PLP) GDC activity in the presence of 0.3 mM coenzyme. Numbers in parentheses indicate number of experiments. Ordinate, GDC activity (in nmoles CO_2/mg protein/h).

15 min. The residue containing synaptosomes and mitochondria (unpurified "synaptosomes") was suspended in 0.1 M K-phosphate buffer (pH 6.5), containing 0.25% Triton X-100 to solubilize the GDC, and was used for manometric determination of GDC activity of the "synaptosomes" [17]. Next, 2 ml of the suspension of "synaptosomes" (about 11 mg protein by Lowry's method [14] in 1 ml) and 0.1 ml of a 0.3 mM solution of pyridoxal-5-phosphate (PLP), from Fluka (Switzerland), or 2 ml of suspension and 0.1 ml of buffer, or 2.1 ml of buffer was introduced into the small containers of a Warburg apparatus. In every case the side retorts contained 0.5 ml of 0.5 M solution of glutamate in the same buffer. The small containers and retorts were flushed through with highly purified N_2 (containing less than 0.001% O_2) for 10 and 2 min respectively. The gaseous phase in the experiment also was N_2. The small containers were kept at a constant temperature for 50 min in the waterbath of a Warburg apparatus, after which the cocks were closed and the reaction started by adding glutamate from the side retort. GDC activity was estimated by the liberation of CO_2 during 1-2 h.

The direct action of TT on glutamate decarboxylase activity of the nerve endings was studied on a highly purified [5, 13] fraction of nerve endings isolated from rat cerebral cortex. Glass bottles containing 5 ml of suspension of synaptosomes in modified [5] Krebs—Ringer solution (1 mg protein in 1 ml) were incubated for 5 min at 37°C with continuous mixing of the suspension with a magnetic mixer, after which 50 µl of TT in 0.1 M Na-phosphate buffer (dose of TT in sample 0.1 or 1.0 MLD in 1 ml) or an equal volume of TT inactivated by boiling, or 50 µl of buffer was then added to the samples. After incubation for 15 min the synaptosomes were separated from the medium by centrifugation (K-24 centrifuge, East Germany; 9000g, 1 min). The supernatant was discarded and the synaptosomes disintegrated by homogenization in 2.5 ml of 0.2% Triton X-100 solution in 0.1 M imidazole-acetate buffer (pH 6.5) in a homogenizer with glass pestle. Glass test tubes containing 2.5 ml of suspension of disintegrated synaptosomes were incubated for 12 min at 27°C. The GDC reaction was started by addition of 2.5 ml of glutamate solution (37°C) in the same buffer. The final concentrations of the components in the sample were: 1 mg protein in 1 ml, 1 mM glutamate, and in some experiments 0.1 mM PLP. Samples of 1 ml of suspension were removed from the test tubes immediately and 5, 10, and 15 min after addition of glutamate and transferred to centrifuge tubes containing 0.5 ml of 15% TCA, and centrifuged in the TsLR-1 centrifuge (1500g, 60 min). The GABA concentration in the supernatants was determined by a modified fluorometric method [12]. In parallel experiments samples taken without incubation (assessment of the initial GABA content in the synaptosomes), samples not containing synaptosomes (reagent control), and blind samples to which GABA was added (final concentration 100 nM; for adjusting the fluorometer) also were treated. Measurements were made on a "Fluorispec SF-100" instrument (Baird Atomic, England) at λ_{ex} = 385 nm and λ_{fl} = 450 nm.

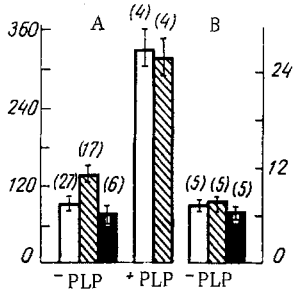

Fig. 2

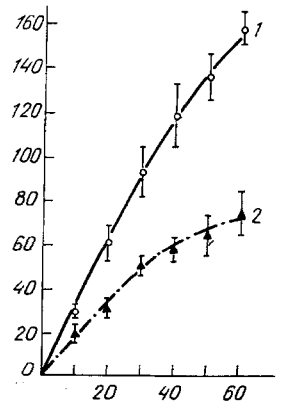

Fig. 3

Fig. 2. Effect of TT on GDC activity of synaptosomes and of partially purified GDC. Shaded colums — GDC activity in presence of 0.1 MLD TT in 1 ml; black columns — the same in the presence of 1.0 MLD TT in 1 ml, unshaded columns — control. A, B) GDC activity of synaptosomes and of partially purified enzyme respectively. Concentration of added coenzyme (+PLP) 0.1 mM. Ordinate, GDC activity (in nmoles GABA/mg protein/h).

Fig. 3. Effect of ATP on activity of partially purified GDC preparation. 1) GDC activity in control, 2) the same in presence of ATP. Abscissa, time (in min); ordinate, GDC activity (in nmoles GABA/mg protein). Concentrations: of glutamate 1.25 mM, of PLP 0.01 mM, of ATP 0.75 mM.

To study the direct action of TT and GDC a partially purified preparation of the enzymes was isolated. For this purpose, 6 g cerebral cortical tissue was carefully homogenized in 54 ml distilled water in a homogenizer with glass pestle. The suspension was centrifuged in a VAC-601 centrifuge (East Germany) at 100,000g for 2 h. The supernatant was dialyzed against 4 liters of 0.1 M imidazole-acetate buffer, pH 6.5, at 4°C for 18 h. The dialysate was made up to a volume of 80 ml with the same buffer, poured into test tubes, and kept at −20°C. On the day of the experiment GDC solutions, 1.3 ml in volume, and containing TT, inactivated TT, or the corresponding volume of phosphate buffer, were incubated for 20 min at 37°C. The reaction was started by addition of 1.25 ml of glutamate solution in the same buffer (37°C) and incubation was continued for another 15 min (final concentrations of components: 1 mg protein in 1 ml; 1 mM glutamate, 2 mM phosphate, dose of TT 0.1 and 1.0 MLD in 1 ml). To study the effect of ATP on GDC activity the samples contained 1.25 mM glutamate, 2.0 mM phosphate, 0.75 mM ATP (from Sigma, USA), and 0.01 mM PFP. Fluorometric determination of the GABA content in the samples was carried out in the same way as for extracts of synaptosomes. To estimate the significance of differences Wilcoson's T test and Student's t test were used.

EXPERIMENTAL RESULTS

Spinal "synaptosomes" decarboxylated glutamate at a slow but constant rate. In experiments carried out in spring the glutamate decarboxylase activity of "synaptosomes" isolated from spinal cord segement poisoned with TT did not differ from the control (Fig. 1B), but in the fall it was significantly ($P_t < 0.02$) higher (about 3 times) than the control (Fig. 1C). Addition of PLP to the samples in a quantity saturating the apoenzyme, led to a considerable increase in GDC activity in the control and in the experiment and also to abolition of the difference noted previously (Fig. 1A). Equalization of the levels of GDC activity after the addition of PLP indicates that the content of the GDC apoenzyme in the synaptosomes is unchanged by the action of TT, but the increase in activity of the poisoned synaptosomes (Fig. 1C) was probably due to greater saturation of the GDC apoenzyme with the coenzyme. It can be concluded from the results of this series of experiments that disturbance of central inhibition by the action of tetanus toxin is not due to inhibition of synthesis of the inhibitory mediator — GABA.

After incubation of synaptosomes isolated from the cerebral cortex with 0.1 MLD TT in 1 ml, a significant ($P_t < 0.01$) increase in GDC activity was found (Fig. 2A). With an increase in the dose of TT in the incubation medium its ability to stimulate GDC activity disappeared and inhibition of the enzyme was observed (Fig. 2A). Just as during poisoning of nerve endings *in vivo*, addition of PLP abolished the difference between the control and experiment (Fig. 2A).

The possibility of a direct action of TT on the enzyme was investigated in experiments with a partially purified preparation of GDC. As Fig. 2B shows, TT did not affect activity of the enzyme. Other arguments had to be found to explain the ability of TT to potentiate GABA synthesis in synaptosomes. The writers postulated previously that the action of TT on GABA synthesis is mediated through changes in the intracellular ATP concentration [7]. It will be clear from Fig. 3 that ATP, in a physiological concentration, inhibits GDC, in agreement with data in the literature [18, 19]. It has been suggested that inhibition of GDC by nucleotides is associated with a decrease in apoenzyme saturation by the coenzyme [18].

In local tetanus potentiation of GABA synthesis in the synaptosomes can be explained either by an increase in concentration of the coenzyme during prolonged excitation of the nervous system, or by partial removal of nucleotide inhibition of GDC as a result of hydrolysis of ATP by Na,K-ATPase [7], whose activity rises considerably in local tetanus [1]. TT does not inhibit oxidative phosphorylation [9], and for that reason inhibition of ATP synthesis can be ruled out.

Potentiation of GABA synthesis during poisoning with TT *in vivo* and *in vitro* can be explained by the ability of TT to induce synaptosomal membrane depolarization [16]. On depolarization of synaptosomal membranes the intracellular ATP concentration is known to fall [15], probably on account of increased utilization of ATP for ion transport and release of ATP from terminals [20], whereas GDC activity rises [15]. In the present experiments depolarization of synaptosomal membranes by an increase in the K^+ concentration in the medium to 56 mM was accompanied by an increase in GDC activity of the synaptosomes by 46%. However, it is not yet clear whether depolarization of the synaptosomal membrane can take place under the influence of a dose of TT as low as 0.1 MLD/ml.

The data described above indicate that TT can induce changes in GABA synthesis through its direct action on nerve endings, thus evidently causing changes in saturation of the GDC apoenzyme with the coenzyme. Disturbance by TT of GABA release from nerve endings is unconditionally not the result of inhibition of GABA synthesis.

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FUNCTIONAL HETEROGENEITY OF CHROMATIN FRACTIONS OBTAINED BY LIMITED HYDROLYSIS OF RAT LIVER NUCLEI BY ENDOGENOUS Ca^{2+},Mg^{2+}-DEPENDENT ENDONUCLEASE

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KEY WORDS: endogenous nuclease; chromatin; transcription; repair.

Fragmentation of chromatin by nucleases is a standard technique which, since the beginning of the 1970s, has achieved widespread popularity for the study of chromatin structure and function [3]. Knowledge of the enzymologic characteristics of the commercial nucleases usually used for this purpose and analysis of the hydrolysis products provide definite ideas on the structural-functional specificity of the chromatin regions which may form fragments. Previously the writers have used endogenous nuclease from rat liver nuclei as the principal enzyme [2, 5, 6]. In this case the use of a nuclease which participates directly in the processes of genome function is particularly interesting [2]. Unfortunately, however, there are no unambiguous data on the functional loads of this enzyme in the literature. As an approach to the study of this problem the writers decided to investigate the functional specificity of chromatin fractions differing in accessibility for endogenous nuclease in the initial stages of hydrolysis (1-3% acid-soluble DNA). Such an approach is justified because, according to our own data [2, 7] and data in the literature [13], the regions into which chromatin is fragmented by endogenous nuclease in the initial stages of hydrolysis are those very regions with which molecules of the enzyme are associated.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats. The method of isolation of the liver nuclei and conditions for activation of endogenous nuclease were described previously [8]. The nuclei were incubated at 37°C and the concentration of nuclear DNA was 6 mg/ml. To fractionate the hydrolyzed chromatin 20 volumes of 0.5 mM EDTA-Na_2, pH 7.0, were added and the suspension was stirred for 30 min on ice. Samples were divided into solubilized (S_1) and nonsolubilized (P_1) fractions by centrifugation at 12,000g (15 min). $MgCl_2$ was added to S_1 up to 2 mM and, after incubation for 30 min on ice, it was centrifuged at 12,000g (15 min). The supernatant fraction was called S_2 and the residue P_2. Fraction S_2 was lyophilized for electrophoretic investigation. Preparation of the DNA samples and the conditions of electrophoresis were described previously [8]. To investigate DNA synthesis, [^{14}C]thymidine was injected into the animals' portal vein 5 min before decapitation in a dose of 100 μCi/100 g. To investigate RNA synthesis, [^{3}H]orotic acid was injected intraperitoneally into the rats 5 min before decapitation in a dose of 30 μCi/100 g. Regeneration of the liver was stimulated by removal of 70% of the organ. Hydrocortisone (from Gedeon Richter, Hungary) was injected intraperitoneally in a dose of 5 mg/100 g 6 h before decapitation. Separation and quantitative determination of DNA and RNA and measurement of radioactivity were carried out as described previously [5].

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