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ULTRASTRUCTURAL AND FUNCTIONAL CHANGES IN RAT BRAIN SYNAPTOSOMES DURING ELECTRICAL STIMULATION IN VITRO

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Electrical stimulation of a suspension of rat brain synaptosomes leads to significant Ca^{++}-dependent liberation of endogenous noradrenalin and to a Ca^{++}-dependent increase in its concentration in the synaptosomes themselves. Cyclic nucleotide phosphodiesterase activity is lowered significantly under these same conditions. No disturbance of synaptosomal ultrastructure is found during stimulation. An increase in the number of electron-dense synaptosomes is observed.

KEY WORDS: ultrastructure of synaptosomes; electrical stimulation; secretion of noradrenalin; phosphodiesterase.

Synaptosomes (isolated nerve endings), isolated from different parts of the CNS of animals, are convenient objects for the neurochemical study of synaptic processes. During depolarizing procedures in vitro – electrical stimulation (ES), an increase in the K^+ concentration, addition of veratrine and ouabain – biochemical changes associated with the Ca^{++}-dependent liberation of neurotransmitters contained in the synaptosomes take place [1, 6].

The object of this investigation was to study the effect of ES of a suspension of synaptosomes on their ultrastructure and also on functional indices such as the rate of Ca^{++}-dependent liberation of noradrenalin (NA) and cyclic nucleotide phosphodiesterase (PDE) activity.

EXPERIMENTAL METHOD

Synaptosomes were isolated from rat brain without the cerebellum by the method described previously [3]. After sedimentation in modified Krebs–Ringer solution (104 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Tris-HCl buffer, pH 7.6, at 37°C) the synaptosomes were suspended in a fresh portion of the same solution (1-3 mg protein/ml and preincubated (15 min, 37°C) with agitation by means of a magnetic stirrer. Part of the suspension was left under the same conditions during ES (control), the rest was subjected to ES by means of platinum ring electrodes at 37°C and with constant

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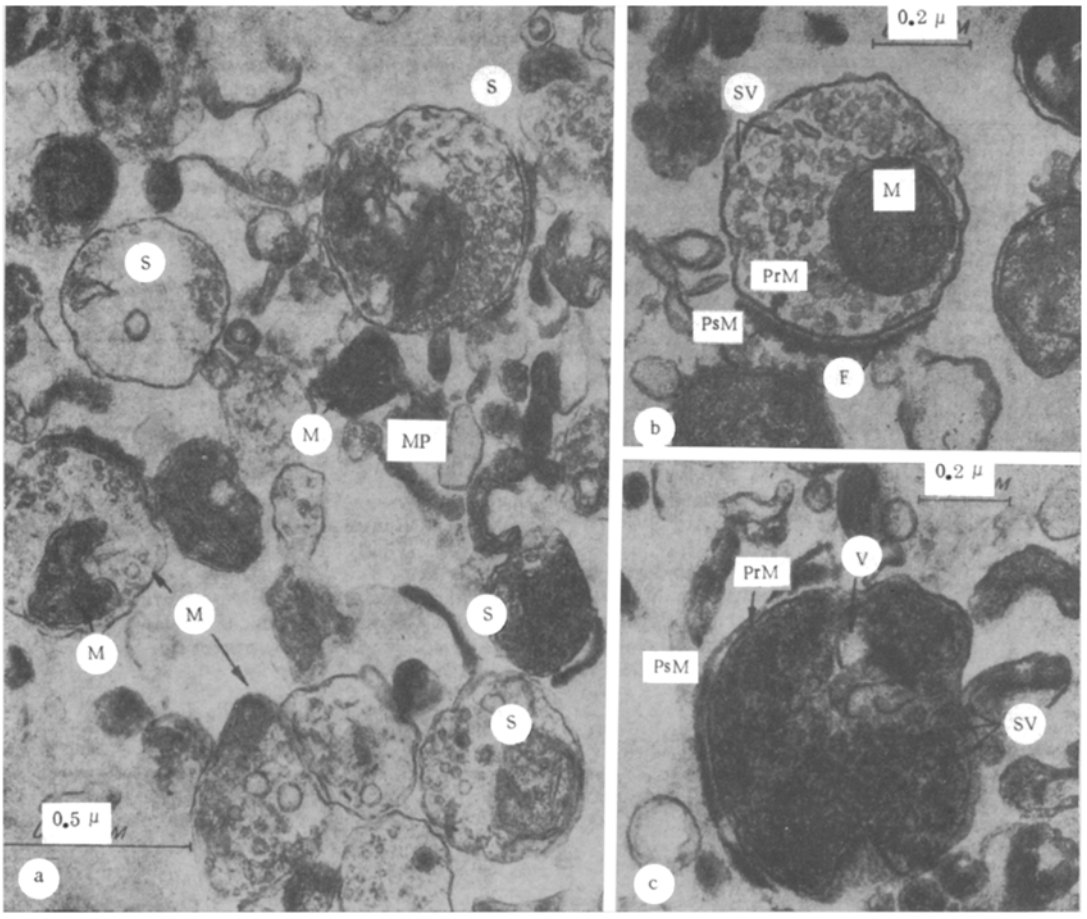

Fig. 1. Synaptosomes of rat brain fixed with formaldehyde in suspension: a) fraction of synaptosomes not subjected to ES, 60,000×. b) individual synaptosomes after ES. 70,000×; c) separate dark synaptosomes of control fraction. 70,000×. S) synaptosomes; M) mitochondrion, MP) membrane profile; SV) synaptic vesicles; PrM) presynaptic membrane; PsM) postsynaptic membrane; F) filament; V) vacuole.

agitation. The diameter of the platinum wire was 1 mm, the diameter of the electrodes and cuvettes 20 mm, and the distance between the electrodes 15 mm. Stimulation was by square pulses of alternating polarity with a frequency of 100 Hz, the duration of a single pulse was 0.4 msec, the interval between paired pulses of different polarity 0.2 msec, and the voltage of the electrodes 7-10 V; the conventional strength of the current, measured by means of a milliammeter graduated for sinusoidal voltage, was 12 mA. An ÉSU-1 electrostimulator was used and the amplitude of pulses of different polarity was measured and strictly equalized as shown by the readings of the oscilloscope. After ES the synaptosomes were separated by centrifugation (20,000 g, 10 min, 4°C). The acid-soluble fraction of the supernatant and the residue of synaptosomes were used for determination of the Na concentration [3]. In experiments in which PDE activity was measured, the residue of synaptosomes was frozen at −10°C and stored for not more than one week. PDE activity in synaptosomes disintegrated in this way was determined by a radioisotope method [4].*

Synaptosomes were fixed for electron-microscopy in experimental and control samples 5-7 sec after the end of ES. Prefixation was carried out in the suspension by adding formaldehyde or OsO_4 up to a concentration of 1% (pH 7.4) [7]. The synaptosomes were then sedimented and, in the case of prefixation with formaldehyde, they were postfixed with 1% OsO_4. Similar procedures also were used to fix the previously sedimented synaptosomes. In all cases the residue was dehydrated and embedded in Durcupan.

Ultrathin sections stained with uranyl acetate and lead citrate by Reynolds' method were examined and photographed in the JEM-7A electron microscope. The state of the vesicular apparatus of the synaptosomes

*This part of the work was undertaken jointly with A. M. Korolev, O. Yu. Frolov, and T. K. Semina (Department of Physiology of Higher Nervous Activity, Faculty of Biology, Moscow University).

was studied in randomly chosen profiles in which the section passed through the synaptic contact. In this way the area of the synaptosomes, their free area (without mitochondria), the relative numbers of different types of synaptic vesicles (SV), and the number of SV per unit free area were determined. The location of the SV in the synaptosomes was noted.

Protein was determined by Lowry's method. Statistical analysis of the results was carried out by the use of the Wilcoxon–Mann–Whitney nonparametric criterion.

EXPERIMENTAL RESULTS

Comparison of the above-mentioned methods of fixation showed that the ultrastructure of the synaptosomes is best preserved after fixation in suspension with formaldehyde. In this case only a negligible fraction of the synaptosomes shows morphological signs of injury, mainly in the form of a disturbance of integrity of the plasma membrane. Only the results of experiments with this type of fixation will be considered from now on. The fraction studied contained not less than 70% of synaptosomes, and also free mitochondria and closed membrane profiles (Fig. 1a). The dimensions of the synaptosomes varied from 0.4 to 0.6 μ, and the usual synaptic organelles – SV, mitochondria, and vacuoles – were observed in them. The mitochondria were small, with few cristae, and their matrix in different synaptosomes differed in electron density. The overwhelming majority of SV were round electron-transparent structures; elongated and coated SV were much less frequently found. As a rule the SV were uniformly distributed in the cytoplasm, but occasionally they were grouped in the center or near the presynaptic membrane. The vacuoles differed in size. Profiles of synaptosomes in which the section passed through the synaptic contact contained fragments of the postsynaptic membrane, sometimes with remnants of the subsynaptic reticulum. The synaptic space had the usual structure: It was filled with electron-dense material, across which sometimes filaments could be seen (Fig. 1b). The matrix of the synaptosomes differed in electron density. On the whole, the synaptosomal ultrastructure was similar to that of nerve endings described during the investigation of brain sections [2].

The data given in Fig. 2 show that ES (for 5 min) of the suspension of synaptosomes led to a considerable and significant increase (by 54%) in the quantity of Na liberated into the incubation medium. This effect was Ca^{++}- dependent: In medium containing 2 mM EGTA instead of Ca^{++}, depolarization did not increase the liberation of this mediator. The NA concentration in the synaptosomes themselves was not reduced after ES, and actually showed a tendency to increase a little, evidence of its more intensive synthesis during depolarization in vitro. PDE activity of the synaptosomes in these experiments also was significantly changed by ES, exposure to which for 1 min led to a marked decrease (by 40%) of its activity. An increase in the duration of ES did not cause any further increase in the effect (Table 1).

Electron-microscopic investigation showed that ES changed neither the number of SV per unit of free area of the synaptosomes nor the geometric dimensions of the synaptosomes or synaptic space. Unlike Jones

TABLE 1. Effect of Electrical Stimulation on Synaptosomal Cyclic AMP Phosphodiesterase Activity

Duration of stimulation, sec	Number of experiments	Specific phosphodiesterase activity, nmoles cyclic AMP/mg protein/min		Change in activity after stimulation, percent
		normal	stimulation	
6	2	18,7 (18,4—19,7)	20,4 (18,6—22,1)	+8,5
60	3	19,7 (18,1—21,3)	12,0 (9,2—15,0)	—39,1*
360	3	19,0 (18,0—19,6)	13,0 (11,2—14,7)	—31,6*
600	2	19,8 (18,5—21,1)	12,7 (11,0—14,1)	—36,9

Legend. Limits of scatter of data representing results of individual experiments indicated in parentheses. Results differing significantly from normal by Wilcoxon–Mann–Whitney criterion ($P < 0.05$) indicated by asterisk.

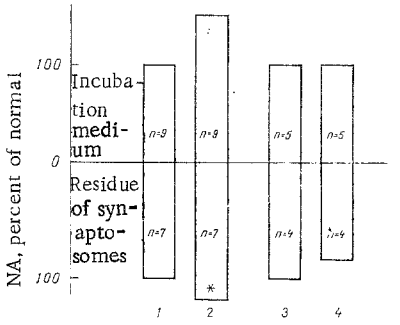

Fig. 2. Effect of electrical stimulation on noradrenalin concentration in incubation medium of synaptosomes and in synaptosomes themselves. 1) Control in presence of Ca^{++}; 2) ES for 5 min in presence of Ca^{++}; 3) control without Ca^{++}; 4) ES without Ca^{++}. Mean data given for results of 5-9 experiments. Asterisk indicates results significantly different from corresponding control ($P < 0.05$). NA concentration in "control in the presence of Ca^{++}" variant made up of: incubation medium – NA, in ng/mg protein of synaptosomal residue, and synaptosomal residue – NA, in ng/mg protein.

and Bradford [7], we were unable to discover any rules governing the distribution of SV within the synaptosomes after ES. The only morphological feature that was significantly changed after ES was the ratio between pale and dark synaptosomes. In the present experiments ES increased the number of pale synaptosomes by 55% (Fig. 1b). This fact may be evidence of the stimulation of biosynthetic processes in the synaptosomes, for their "pale" and "dark" state can evidently be regarded as analogs of the corresponding state of the cell found in the number of organs during their functioning.

On the whole these investigations show conclusively that the Ca^{++}-dependent liberation of NA by the synaptosomes observed in response to ES was not due to a disturbance of their ultrastructure. The fact that we were unable to discover any preferential accumulation of SV near the active zone suggests the possibility of a nonvesicular liberation of NA during ES. Meanwhile it must be remembered that the process of fixation of synaptosomes can itself distort the structural picture caused by depolarization. The vesicular nature of the NA liberated under these conditions is supported by data showing that besides NA, the ATP contained in SV also is liberated [3], and also by the results of preliminary experiments in which imipramine, an inhibitor of the nonvesicular liberation of NA, did not reduce the effect of depolarization.

In 1970, Rasmussen [9] suggested on a priori grounds that the accumulation of cyclic AMP in the synaptoplasm during excitation of nerve endings can give rise to phosphorylation of contractile proteins participating in the secretion of mediators. However, it was later shown that a rapid increase in the cyclic AMP concentration in nerve endings does not arise during depolarization of synaptosomes [5] or of brain sections [8]. Nevertheless, our data showing inhibition of PDE activity during short-term ES point indirectly to the possibility of substantial changes in the cyclic AMP system in depolarized synaptosomes.

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