Expression of Adenylyl Cyclase Type IX and Calcineurin in Synapses of the Central Nervous System

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Distribution of type IX adenylyl cyclase and protein phosphatase calcineurin in the brain and in cultured hippocampal neurons from albino rat was immunohistochemically studied. Both enzymes were detected simultaneously in all synaptic structures of most cerebral neurons except for presynaptic sites, where calcium-inhibited type IX adenylyl cyclase was absent.

Key words: *synapse*; *adenylyl cyclase type IX*; *calcineurin*; *immunohistochemistry*

Synaptic plasticity, or different efficiency of synaptic transmission, is the central point of the modern concept on the central nervous function. The key role in signaling pathways is played by adenylyl cyclase (cAMP-mediated) and protein phosphatase calcineurin (Ca^{2+}/calmodulin-mediated) [1,14]. There are 9 isoforms of adenylyl cyclase (AC) differing in regulatory properties and localization in brain structures [13]. The distribution of adenylyl cyclase isoforms is usually studied by *in situ* hybridization, only few isoforms are examined by immunohistochemical methods [5,6]. It is important to detect distribution of AC isoforms in the pre- and postsynaptic parts of the neuronal contacts, because activities of these enzymes and their function as molecular detectors of external stimuli are responsible for synaptic plasticity [7].

Type IX AC (AC-IX) is the most widespread AC isoform in the brain. It belongs to the subgroup of calcium-inhibited AC; the inhibitory effect of calcium ions is mediated by Ca^{2+}-activated calcineurin [4,10]. Calcineurin is the major form of protein phosphatases in the central nervous system. Being involved in the regulation of many enzyme systems, calcineurin plays a vital role in the realization of postsynaptic processes and is also considered as a product of memory suppressor gene [3]. Taking into account close functional interrelation between AC-IX and calcineurin, it is interesting to analyze the distribution pattern of these enzymes in pre- and postsynaptic structures.

Our aim was to study localization of type IX adenylyl cyclase and calcineurin in the brain and cultured hippocampal neurons from albino rats using histochemical methods.

MATERIALS AND METHODS

Experiments were carried out on 12 male Wistar albino rats weighing 200-250 g. The animals were narcotized with hexenal (40 mg/kg) and perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. The brain was removed and fixed for 6-8 h in 4% paraformaldehyde with 0.025% glutaraldehyde for electron microscopy. Vibrotome sections (50-60 μ) and cryostat (8-12 μ) sections were prepared (cryoprotection in 15 and 30% sucrose). Vibrotome sections were used for electron microscopy.

Hippocampal neurons for primary culture were isolated from 2-day-old rat pups [10]. On day 12 of culturing the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (20 min) and incubated in 100% methanol (-10°C, 10 min).

Original antibodies to a unique amino acid sequence at the N-end of AC-IX molecule were used as

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anti-AC-IX antibodies; their specificity was verified by immunoblotting and immunohistochemistry using transfected HEK293 cells characterized by a high AC-IX expression. We also used commercial antibodies to microtubule-associated MAP2 (1:500) and *tau* (1:300) proteins, synaptic vesicle protein synaptophysin (1:800, *Sigma*), and calcineurin catalytic subunit (1:100, *Transduction Laboratories*). Biotin- and fluorochrome-bound antibodies Alexa 594 and 488 (1:150, *Molecular Probes*) were used as secondary antibodies. Immunohistochemical reaction was carried out routinely, according to manufacturer's instruction. Preparations were examined under a luminescent microscope equipped with Digital Pixel image deconvolution system and under a Zeiss LSM 510 confocal microscope.

Sections for electron microscopy were stained using avidin/biotin immunoperoxidase techniques, contrasted with osmium, dehydrated, and embedded in Epon-Araldite. The ultrathin slices were contrasted with lead citrate and analyzed under a EM-125 electron microscope.

RESULTS

Immunohistochemical analysis revealed expression of AC-IX in most neurons in all tested brain regions. Double immunofluorescence staining with antibodies to AC-IX and MAP2 (a marker of the dendrosomatic compartment) showed that all MAP2-positive neurons expressed AC-IX. Localization of AC-IX-specific immunoreactivity in the brain was consisted with the results obtained by *in situ* hybridization reflecting distribution of AC-IX mRNA [4]. The most pronounced immunoreactivity was found in large apical dendrites of pyramidal neurons of the cerebral cortex and Ammon's horn. The pattern of calcineurin expression mostly coincided with the localization of AC-IX except some AC-IX-immunopositive neurons in the hippocampus containing no calcineurin. These neurons could be attested to calcineurin-lacking interneurons found earlier in the hippocampus [12].

Double immunofluorescence staining with antibodies to AC-IX and *tau* protein (*in situ* detected only in axons) revealed the presence of both these proteins in neurites, in particular, in large myelinated axons.

Double immunostaining for AC-IX and synaptophysin (a presynaptic marker) showed that AC-IX is absent in the presynaptic terminals. These findings were confirmed in the study of large synaptic glomeruli in the cerebellar cortex granular layer (Fig. 1) and synapses formed on rarely distributed large neurons in the brain stem. Unlike AC-IX. calcineurin was located in presynaptic terminals (Fig. 1).

Ultrastructural electron microscopic examination of AC-IX expression in the cerebral cortex and hip-

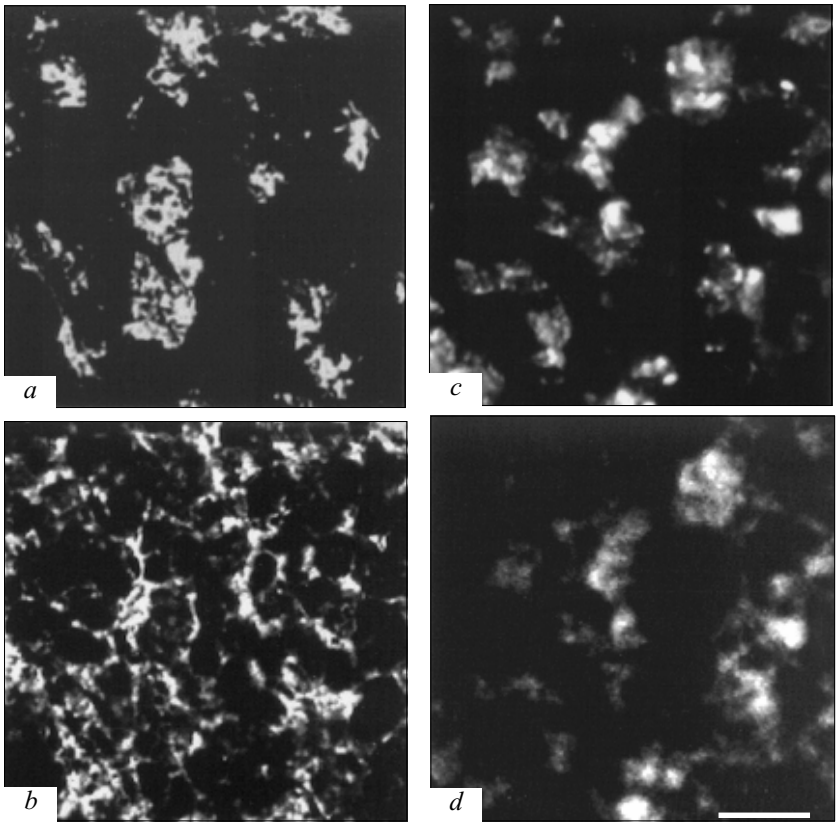

Fig. 1. Synaptic glomeruli in the granular layer of rat cerebellar cortex. Double immunofluorescence staining with antibodies to synaptophysin and adenylyl cyclase type IX, (*a*, *b*), and synaptophysin and calcineurin (*c*, *d*). Superposition of fluorescence areas (*a* and *b*) indicates the absence of adenylyl cyclase in presynaptic terminals. Synaptophysin-positive presynaptic varicosities contain calcineurin. Confocal microscopy. Secondary antibodes labeled with Alexa 594 and 488 fluorochromes. The mark corresponds to 10 μ.

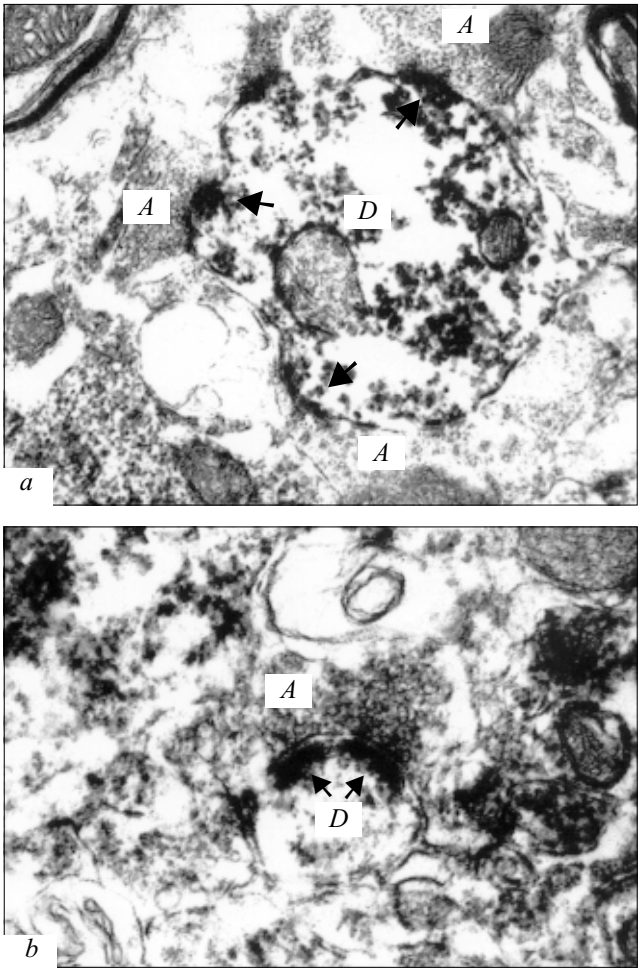

Fig. 2. Ultrastructure of synapses in the hippocampus (*a*, ×19,000) and cerebral cortex (*b*, ×20,000). Immunohistochemical localization of adenylyl cyclase type IX. No immunopositive material in presynaptic axon profiles. Electron dense areas in postsynaptic profiles are seen near active zones (arrowheads). *A*: axon, *D*: dendrite, synaptic contacts (arrows).

pocampus revealed immunopositive electron dense matter only in postsynaptic axon profiles (Fig. 2), while calcineurin was detected in both postsynaptic and presynaptic terminals.

Experiments with cultured neurons confirmed the absence of AC-IX in the presynaptic compartments. Thus, double staining with antibodies specific for AC-IX and synaptophysin indicated that the axon parts, which were immunopositive to synaptophysin, exhibited no fluorescence specific for AC-IX (Fig. 3). By contrast, double staining with antibodies synaptophysin and calcineurin revealed the presence of both peptides in the same structures.

The distribution of different AC isoforms in the brain indicates that AC-IX is uniformly distributed in the brain and is present in most if not all neurons. AC-IX is present in all neuronal elements except presynaptic terminals. Interestingly, AC-IX was found even in preterminal part of the axon and its branches, but not in the presynaptic profiles. It was previously shown that presynaptic bulbs in motor nerve terminals contain no AC-IX [2]. Such peculiarities of AC-IX localization remain unclear. It should be noted, that only one type of AC was detected in presynaptic structures: calcium-inhibited AC type I [8], which is also localized in the postsynaptic compartments.

Calcium-activated AC in synapses is primarily involved in activation of cAMP, proteinkinase A, and activation of signal transduction cascades, resulting in conformational changes of ion channels and transcription of early response genes [11]. The role of calcium-inhibited AC in synapses is still poorly understood. It can be hypothesized that these enzymes (in particular, the most widespread isoform AC-IX) serve as a buffer preventing sharp changes in intracellular cAMP level and maintaining it at a relatively constant value.

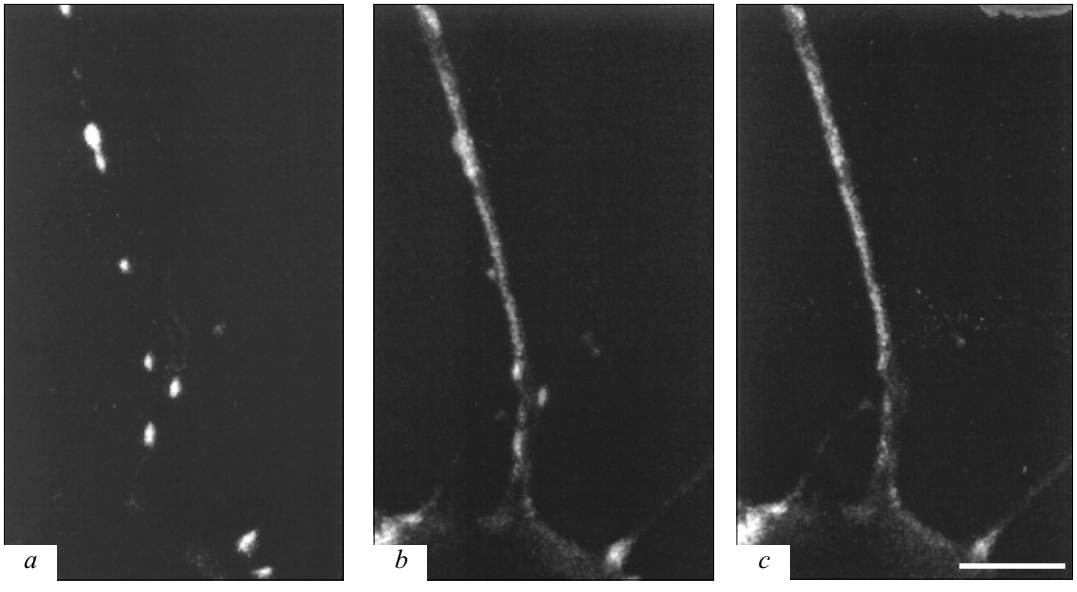

Fig. 3. Primary culture of hippocampal neurons. Double immunofluorescence staining with antibodies to synaptophysin and type IX adenylyl cyclase. No overlapping fluorescence zones. Confocal microscopy. Secondary antibodies labeled with Alexa 594 and 488 fluorochromes. The mark corresponds to 20 μ.

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