HISTOCHEMISTRY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN NERVOUS TISSUE. IV. IMMUNOCYTOCHEMICAL INVESTIGATIONS

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Cyclic nucleotide phosphodiesterase (PDE) was demonstrated by an immunocytochemical method on the electron microscopic level. The method was chosen as a preembedding PAP-technique with osmification of the diamino benzidine (DAB) reaction product. Antigenic sites of PDE were detected in synapses, on endoplasmic reticulum, microtubules, the nuclear envelope, the cytoplasm and processes of distinct neurons and glial cells. Only a little reaction product was found in the capillary endothelium. The morphology was better in comparison to enzyme histochemical methods. There was a good agreement between the localizations of the enzymatic reaction (16) and the antigenic sites of the PDE on the electron microscopic level.

The exact localization of the cyclic nucleotide degrading system is of great interest for the understanding of cyclic nucleotide mediated processes. Therefore a number of investigations was carried out to localize the PDE (2, 3, 5, 11, 18) and to clarify the function of the isoenzymes (7, 9, 21). Because of the several isoenzymes involved, this system is not easy to understand and many conclusions are speculative (9, 12, 20, 21). Two of these isoenzymes, the Ca/calmodulin activated and the cGMP activated isoenzyme, could be detected by an antiserum, prepared by Poeggel *et al.* (14). On the light microscopic level there was a nearly complete agreement between enzymatic reaction and antigenic sites of the PDE (15). To overcome the bad tissue preservation using the enzyme histochemical method (16) the PDE protein should be localized by an immunocytochemical technique.

MATERIAL AND METHODS

The antiserum was prepared as described previously (14). Adult male albino rats were anesthetized and perfused for 15 min with a mixture of freshly prepared 4% paraformaldehyde and 0.5% glutaraldehyde in 100 mM phosphate buffered saline pH 7.4 (PBS). After removing the brain, the hippocampus was prepared and fixed for a further 2 hr in the same mixture. Following the fixation, the hippocampus was

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FIG. 1. Immunocytochemical demonstration of PDE in the synaptic region, stratum radiatum of the rat hippocampus.

- a. $\times 77,000$ No poststaining; strong reaction of the postsynaptic thickening.
- b. $\times 144,000$ No poststaining; reaction of pre- and postsynaptic membranes.
- c. $\times 51,000$ No poststaining; note the reaction in the glial cell process surrounding the synaptic region.

cut into 50 μm slices with an Oxford Vibratome and the slices were washed overnight. The next day the slices were rinsed for 30 min in a 1% solution of $\text{Na}(\text{BH}_4)$ and washed for a further 30 min with PBS. The immunohistochemical procedure was made according to Sternberger (19), followed by a 60 min osmification with 1% OsO_4 at 4°C. The slices were then rinsed, dehydrated with graded acetone and embedded in Durcupan. Ultrathin sections were cut with a Reichert ultramicrotome and evaluated with a Tesla BS 500 electron microscope. The antiserum was diluted 1 : 1000, the specificity of the reaction was controlled by comparison with the preimmunoserum.

RESULTS

Antigenic sites of the PDE were detected in specific neurons and glial cells. A strong reaction product was found in the postsynaptic densities (Figs. 1a, b, c) of axo-dendritic synapses. The enzyme protein was also detected on the presynaptic membrane (Fig. 1b). Not all synapses were reactive. In the hippocampus of the rat, the reaction of neurons was limited to pyramidal and granular cells and their processes. These neurons had a strong labelling of the surfaces of mitochondria, nuclei and endoplasmic reticulum (Fig. 2). Furthermore, antigenic sites were detected on microtubules of dendrites (Fig. 3) and in axons (Fig. 4). Glial cell processes were very reactive (Fig. 1c, 5), especially astrocyte processes surrounding capillaries (Fig. 6). The capillary endothelium showed only little, if any reaction. Controls were carried

FIG. 2. Detection of PDE antigenic sites in a pyramidal cell of the CA2-field of the rat hippocampus. Note the labelling of the surfaces of the nucleus, mitochondria and endoplasmic reticulum. $\times 60,000$, no poststaining.

FIG. 3. Reaction of dendrites of hippocampal pyramidal cells. Note the strong microtubular staining.

- a. $\times 36,000$ No poststaining, stratum radiatum (CA 3-field)
- b. $\times 44,000$ No poststaining, stratum radiatum (CA 3-field)
- c. $\times 26,000$ No poststaining, stratum oriens (CA 3-field)

FIG. 4. PDE-like immunoreactivity in an axon. No poststaining, $\times 100,000$.

out with the preimmunoserum and were consistently negative (Fig. 7).

DISCUSSION

All authors, who have published studies about PDE in nervous tissue, using histochemical methods in nervous tissue localized the enzyme exclusively in synapses (15). We found the same synaptic localization of the PDE with enzyme histochemical methods as well as with immunocytochemical methods (16) (Figs. 1a, b, c). Grab *et al.* (8), described in postsynaptic densities two PDE-isoenzymes, a calmodulin independent and a calmodulin dependent enzyme. Ariano and Appleman (3) found one aldehyde resistant isoenzyme in synapses which was Ca/calmodulin activatable. They localized the enzyme in asymmetric axospinous terminals in the striatum and neocortex. This result is in agreement with our synaptic localization (Fig. 1a, b). The neuronal reaction only in pyramidal and granular cells of the hippocampus was also found by Kincaid *et al.* (11), who localized the Ca/calmodulin activated isoenzyme with specific antibodies in distinct neurons. In contrast to Kincaid *et al.* (11), the Purkinje cells of the cerebellum showed only a little reaction with our antibody, but we found a reaction of glial cell processes surrounding the Purkinje cells (14). In agreement with biochemical (17, 22) and enzyme histochemical methods (16), a strong reaction with antigenic sites on microtubules was detectable (Fig. 3). According to Runge *et al.* (17), this is an intrinsic Ca/calmodulin activated phosphodiesterase, probably integrated into the cyclic nucleotide regulated phosphorylation of microtubular proteins. Similar processes could be reflected by the membrane associated localization of PDE on the surfaces of intracellular organelles, a participation of the enzyme in the

FIG. 5. PDE-like immunoreactivity in a glial cell process. No poststaining, $\times 77,000$ Stratum moleculare of the CA 3-field.

FIG. 6. Strong PDE-like immunoreactivity in astroglial processes surrounding a capillary. The endothelium cell is not stained. $\times 20,000$ No poststaining, stratum pyramidale of the CA 3-field.

FIG. 7. Control of the specificity of the immunologic reaction. Instead of the antiserum the preimmunoserum of the same rabbit was used. $\times 50,000$

control of the phosphorylation status of membrane proteins. In contrast to Ariano (1), we were not able to find, neither with enzyme histochemical methods using the specific cerium technique (16) nor with the immunocytochemical technique, a distinct PDE reaction in the capillary endothelium. However, a strong reaction using both techniques was found in the astrocyte processes (16) (Fig. 6). Chan-Palay and Palay (4) described a positive reaction for cyclic GMP in glial cells surrounding capillaries, whereas the endothelium showed no reaction. According to Hidaka *et al.* (9) the cyclic GMP hydrolysis is mainly controlled by the Ca/calmodulin activated isoenzyme of the PDE. That is why the reaction in glial cell processes could be due to this isoenzyme. Shanta *et al.* (18) could also detect a glial cell localized PDE activity. Glial cell processes in the area surrounding of synapses were regularly stained using our procedure (Fig. 1c). These findings support the important role of the glial cells in realizing the homeostasis in the brain. Generally, our enzyme histochemical results are in agreement with our immunohistochemical results. The advantage of the immunotechnique is a better tissue preservation.

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