Scanning Microscopy

Volume 8 | Number 2 Article 16

6-12-1994

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Scanning Microscopy, Vol. 8, No. 2, 1994 (Pages 337-343) 0891-7035/94$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

DISTRIBUTION OF Ca-ATPases IN THE MEDIAL HABENULA IN MOUSE

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(Received for publication January 31, 1994 and in revised form June 12, 1994)

Abstract

The aim of this study was to investigate the distribution of the ecto-Ca,Mg-adenosine-triphosphatases (ecto-Ca,Mg-ATPases) in the medial habenular nucleus. Nerve terminals that seemed to be similar in morphological terms showed a different distribution of enzyme activity. Also, synapses showed a different distribution of enzyme activity. This could be related to the involvement of different neurotransmitters and modulators.

Key Words: Ecto-Ca-adenosine-triphosphatase (ecto-Ca-ATPase), Ca-pump, medial habenula, neurotransmission, neurotransmitters, nerve terminal, synapse.

Introduction

Several Ca-stimulated adenosine-triphosphatases (ATPase) activities have been reported, mostly in biochemical studies, in the central nervous system (CNS) but not specifically in the habenula. These Ca-ATPases may exhibit high or low affinity for calcium in the presence or absence of magnesium, and most of them are related to the active transport of calcium (6, 7, 26, 34, 36, 43, 44). These ATPases are involved in the maintenance of low Ca concentrations in the nerve cells in the face of high extracellular concentrations. Other membrane-associated Ca,Mg-ATPases are ectoenzymes, but their role has not been clarified yet (10, 19, 24, 30, 31, 41). It is suggested that, because they hydrolyze ATP at the exterior face of the plasma membrane, they are involved in the type of neurotransmission where ATP is a fast neurotransmitter (51, 52).

The habenula is potentially an important link between the forebrain and midbrain. Although its physiological role has been investigated for many years (22, 23), interest in its role in neurotransmission has only risen during the last few years.

The medial habenular nucleus consists of one type of bushy cell with axons entering the habenulopeduncular tract. At the electron microscopic (EM) level described by Tokunaga and Otani (49), these bushy neurons make asymmetric contacts which contain clear vesicles of spherical shape. Other bushy neurons with clear pleomorphic vesicles make symmetric contacts (both on dendrites). There are two kinds of large, dense-core vesicle-containing terminals, but these have not yet been identified. Since 1978, immunocytochemical and *in situ* hybridization investigations have clarified the presence of special enzymes, such as, 2C protein phosphatase (1), P-type Ca-channels (16), and several neurotransmitters (18, 25, 17) and neurotransmitter receptors, e.g., AMPA-selective glutamate receptors (27, 35), nicotinic acetylcholine receptors (18, 28, 29, 33), GABA-A and B receptors (9) and, for the first time in the CNS, ATP receptors in the medial habenular nucleus (11). In view of this, a different distribution or amount of Ca-ATPases

in the medial habenular nucleus in proportion to other areas in the brain would be of great importance.

Our purpose was to determine the ultra-cytochemical localization of the ecto-Ca,Mg-ATPase activity in this system using an enzyme-histochemical method. Although the lack of specific inhibitors of ecto-ATPases prevents a more precise determination of their identity, they can be distinguished from Ca-pump ATPases, being resistent to Ca-pump inhibitors, e.g., quercetin. We propose that our results represent the activity of these ecto-Ca,Mg-ATPases involved in neurotransmission.

Materials and Methods

We used a modification of the enzyme histochemical method of Salama *et al.* (42).

Male albino mice were used throughout this study. Animals were anaesthetized by nembutal and then perfused through the ascending aorta with ice-cold 3% paraformaldehyde, 1% glutaraldehyde and 0.25 M sucrose in 0.05 M cacodylate buffer (pH 7.4) for 30 minutes. The brain was quickly removed, and a 2-3 mm thick tissue block was postfixed in a new portion of the same solution for 15 minutes at 0-5°C. Vibratome sections were cut at 60 μm and washed in cacodylate buffer with 0.25 M sucrose (pH 7.4) for 45 minutes at 0-5°C.

Sections were incubated in a medium containing Tris-maleate buffer (70 mM, pH 7.2), ATP (1 mM), $CaCl_2$ (3 mM), $CeCl_3$ (2 mM), $MnCl_2$ (5 mM), and levamisole (5 mM) for 1 hour at room temperature. Following rinses (3 x 10 minutes) in the buffer, postfixation with 1% osmium in cacodylate buffer for 30 minutes, and pre-embedding counter-staining with 2% uranyl acetate in 70% ethanol for 1 hour, the sections were embedded in Araldite 6005.

Ultrathin sections were examined with a JEOL JEM 100C transmission electron microscope operated at an accelerating voltage of 70 kV.

Controls were used to demonstrate the specificity of the reaction product: (**a**) $CaCl_2$ was deleted and 10 mM ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N-tetra-acetic acid (EGTA) added to the reaction mixture; and (**b**) ATP was omitted from the incubation medium.

To confirm that the reaction product did not represent Na,K-ATPase or the Ca-pump, parallel experiments were carried out in the presence of 1 mM ouabain (inhibitor of Na,K-ATPase) or 1 mM quercetin (Ca-pump inhibitor). Chemicals were obtained from SIGMA.

Results

At the EM level, the Ca-ATPase reaction product was seen with a punctate-linear distribution outlining most plasma membranes of nerve terminals, dendrites and glia cells as well as in the synaptic clefts (Fig. 1a). We found significant differences within the examined brain region. Some parts of the investigated area showed intense ecto-ATPase activity, but, in other parts, although they seemed to be morphologically similar, this activity was either much less or absent (Fig. 1b). We also found ATPase activity in some glia cells. This activity decreased but did not disappear in the presence of quercetin. The myelin of the axons was found to be only weakly labelled (Fig. 1c).

In contrast to the results of Mata and Fink (26), who found Ca-ATPase activity on the surface of synaptic vesicles, most synaptic vesicles were unlabelled whether or not quercetin was present. The labelled terminals were near the labelled glia cells (Fig. 1d). On the other hand, terminals and synaptic contacts, devoid of precipitates, could also be observed (Fig. 1e).

We never found a reaction product in the neuronal perikaryon (not shown).

Control sections assayed in the absence of the substrate ATP or in the presence of EGTA plus ATP, when $CaCl_2$ was omitted from the incubation medium, demonstrated no reaction product (Fig. 1f). In the presence of the Na,K-ATPase inhibitor ouabain, the distribution of Ca-ATPase activity appeared essentially the same as without ouabain (Fig. 2a). Quercetin decreased the amount of the reaction product in some areas, but the presence of the deposit in the synaptic cleft was unaffected (Figs. 2b and 2c).

Discussion

The method most often used for localization of Ca-ATPase activity is a lead-precipitation technique developed by Ando *et al.* (2). We used cerium ions as capturing agents since the use of cerium has several advantages (50). The delicate reaction product allows a very precise localization and makes this method especially suitable to show enzyme activities on the surface of nerve terminals where the evaluation of a coarse precipitate would be difficult. The method works under nearly physiological conditions (the pH is 7.4 instead of 9 as in methods using lead as a capturing agent), is highly reproducible, and cerium has a less inhibitory effect on at least a number of enzymes (39, 40).

We found Ca,Mg-ATPase reactivity in almost every case on the external surface of the nerve terminals and glia cells and, very seldomly, in some fields only within the glia cells and on the synaptic vesicles in the nerve terminals.

Although the direct enzyme-histochemical method for Ca-ATPase is not able to distinguish Ca-transporting ATPases from ecto-ATPases (45), our results undoubtedly show "ecto" localization on the terminals, and this

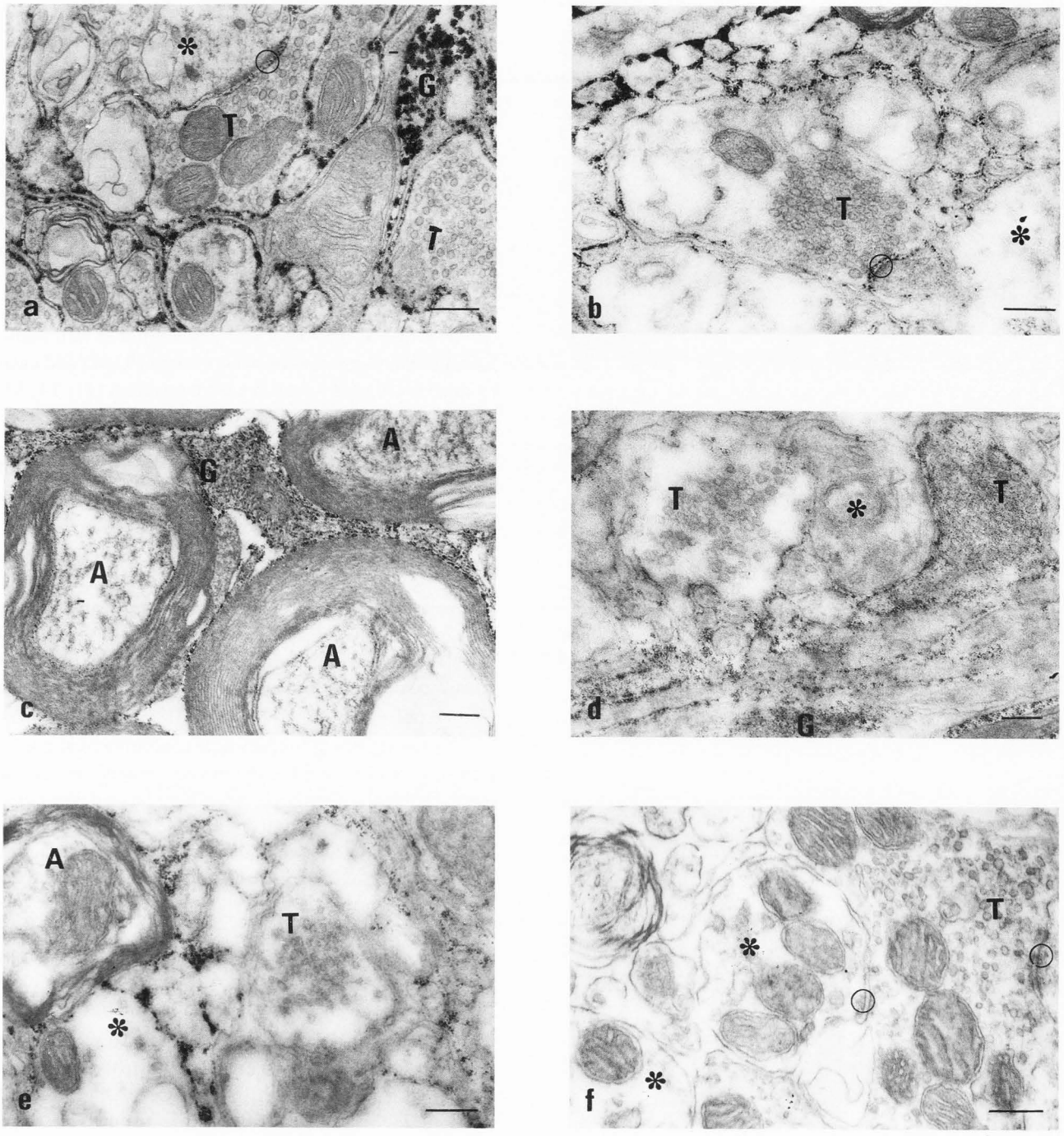

Figure 1. Electron micrographs showing distribution of Ca-ATPase activities in the medial habenula of the mouse. The reaction product is cerium phosphate. **a.** The deposit appears on the external surface of the membrane of nerve terminals (T), dendrites (asterisk) and on the surface of other elements of the neuropil as well as in some glial cell (G). Some nerve terminals display uniform, others display different types of vesicle. The large dense-cored vesicles probably contain ATP. **b.** Distribution of Ca-ATPase activity is uneven in this region although no morphological difference can be seen. The circle shows an asymmetric synapse. **c.** Heavily stained glial cell (G) among the weakly labelled axons (A). **d.** Two similar nerve terminals (T) with different activity of Ca-ATPase. In one terminal, vesicles also show enzyme activity. A heavily stained glial cell (G) can also be seen. **e.** Nerve terminal, with stained membranes nearby, devoid of any precipitate. **f.** Control reaction without the substrate ATP. No reaction product visible. The result is similar when we used EGTA plus ATP. Asterisk: dendrite; G: glial cell; T: nerve terminal; circle: synapse. Bars = 0.25 μm.

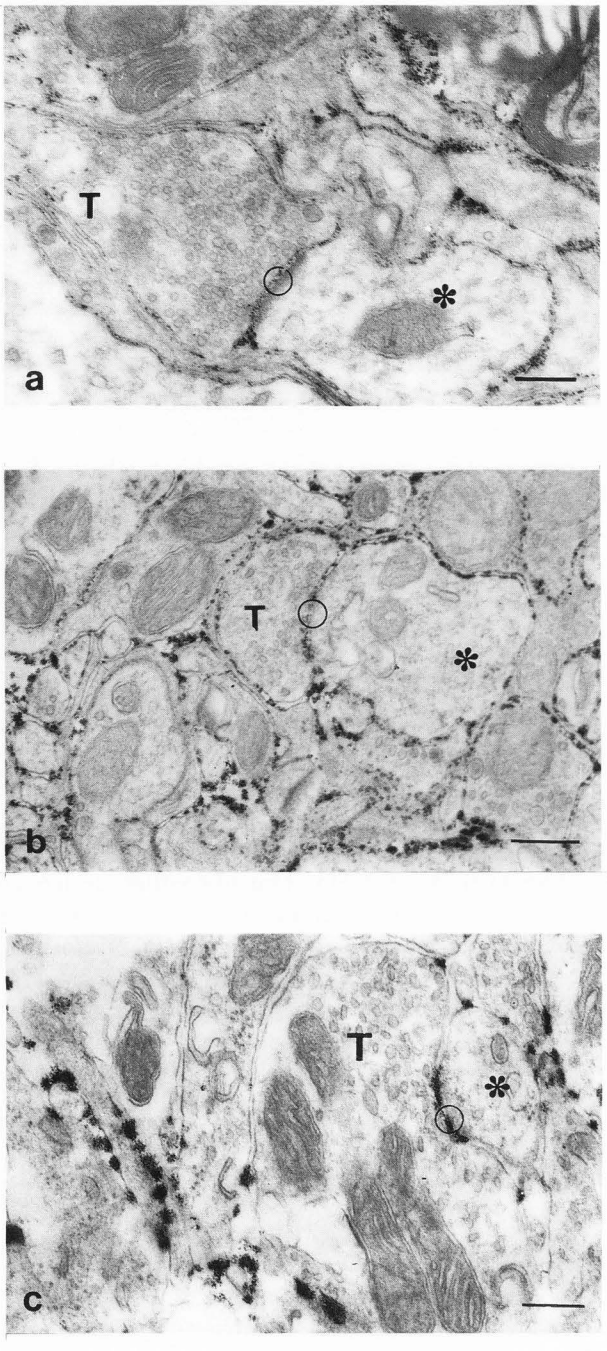

Figure 2. Ca-ATPase activity in the presence of two inhibitors. **a.** The Na,K-ATPase inhibitor ouabain did not change the amount or distribution of enzyme activity. **b-c.** Use of the Ca-pump inhibitor quercetin. **b.** In this region, there is no effect. The amount and distribution of enzyme activity seems to be unaffected. **c.** In this part of the section, the enzyme activity decreased, but, in the synapse, it remained the same. Asterisk: dendrite; G: glial cell; T: nerve terminal; circle: synapse. Bars = 0.25 μm.

activity did not disappear after the use of a Ca-pump inhibitor. In some studies (12, 13, 21, 26, 32, 36, 56), the authors also show some ecto-ATPase activity in morphological terms, but they call it either just membrane-associated ATPase or Ca-pump and suggest these to be involved in Ca mobilization and regulation or in cell maturation. Other authors report ecto-ATPase activity and attribute to it a role in maturation (54), in cell adhesion (3, 8, 24), in Ca metabolism and mobilization (15, 19, 24, 45), and in the inactivation of the neurotransmitter ATP (4, 10, 20, 53).

The role of ATP as a neurotransmitter is well established in the peripheral nervous system (5, 51) and even in some areas of the central nervous system (11, 14, 37, 38, 48, 55). In this paper, we wanted to localize the enzyme that can metabolize ATP in the synapses, and, therefore, the ecto-enzyme activity on the surface of glial and endothelial cells is outside the scope of the present paper.

The uneven distribution of this enzyme (Fig. 1b) is not unusual. Soji *et al.* (43) suggest that membranous enzymes are not uniformly distributed over all pituitary cells, but rather, they are specific for a given cell population(s). Purine-catabolizing enzymes, such as adenosine desaminase, have a similar uneven distribution (47).

The terminals of the habenula contain several neurotransmitters (49), and we did not see enzyme activity in every terminal. Terminals containing large dense cored vesicles, the presumed intracellular ATP stores, were always stained. However, stained terminals containing other types of vesicles were also found (Figs. 1a and 2a-2c). Nerve terminals and synapses which were devoid of deposits (Fig. 1e) may correspond to the non-purinergic neural contacts such as glutamatergic or GABAergic synapses. Further investigations are necessary to identify the terminals and their neurotransmitters.

This enzyme-histochemical method is not suitable to distinguish between ecto-enzyme isoforms as was done with *in situ* hybridization in the case of the Ca-pump (46), but the results suggest to us that the enzyme activity obtained in the presence of a Ca-pump inhibitor, the ecto-ATPase activity, is involved in the rapid metabolization of neurotransmitter ATP.

Acknowledgements

We are grateful to Dr. Erno Bácsy for his help with the preparation of the manuscript. We also wish to thank Ms. Katalin Lengyel and Gábor Terstyánszky for their expert assistance. This work was supported by OTKA Grant Nr. 2917.

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Discussion with Reviewers

W.C. de Bruijn: There is some vital information lacking in the **Materials and Methods** section with respect to the treatment given to the tissue. Especially with respect to: (**a**) the omission of the osmium post-fixation step, and the use of unstained ultrathin sections, and/or light microscopical studies about the distribution of the precipitate within the vibratome sections; (**b**) the use of any pre-incubation with media without substrate to ensure a sufficient distribution in the tissue of the catch Ce ions; and (**c**) the addition of Ce ions to the rinse media.

As the author mentions the paper by Van Noorden and Frederiks (1993), conditions can be found there and in additional papers about the use of cerium which are now lacking.

Author: Thank you for your comments. In the beginning, I carried out the experiments as described in previous papers, and, of course, I examined ultrathin unstained sections. There was no difference in the distribution of precipitate between unstained and postfixed sections. As mentioned in the text, I used uranyl acetate staining in blocks only in order to avoid the rearrangement of the deposit. For the sake of a better ultrastructural preservation of the tissue, I tried to minimize the incubation time. After this fixation (3% paraformaldehyde and 1% glutaraldehyde) both Ce and the much larger molecule ATP can penetrate the membranes. I omitted, therefore, the preincubation step and did not feel that it was necessary to use cerium in the rinse media.

W.C. de Bruijn: Irregular precipitation is a problem that has to be tackled by steps proposed in the literature. In the **Discussion**, the problem of "presence" as compared to "distribution" is insufficiently worked out due to this problem. The use of the inhibitors is theoretically acceptable, but an explanation in terms of quantitative parameters has to be omitted, as such parameters are not measured and are only interpreted subjectively, which is fatal with respect to the problem of irregular distribution.

Author: Since numerous parallel experiments were performed and the phenomenon of "irregular distribution" was always the same, I think that it is not an artifact but a characteristic feature of that area of the medial habenula. Unfortunately, I did not find such an anatomical description of this area at the EM in the literature which could help me to clarify this question. And, because at present, there is no antibody against ecto-ATPases, I had to assess enzyme specificity by inhibiting other types of ATPases. I hope that further experiments which can identify those regions, will be able to give an exact answer to this question.

K.H. Körtje: Did you check the calcium dependence of the ATPase activity? What is the effect of replacing calcium ions by magnesium ions? Did you observe ATPase activity without additional calcium and without addition of EGTA, in other words, ATPase activity which is activated by low calcium concentrations?

Author: Yes, I did all that. The calcium was replaced by magnesium or manganese ions, and I found some ATPase activity. Without calcium, some traces of ATPase activity could also be seen. I agree with your suggestion that this activity probably belongs to the high Ca affinity ecto-ATPase(s).

T. Chiba: The significance of the reaction products in the cytoplasm of the glia cells is not discussed. The deposits are also seen in association with the axolemma of intervaricose or preterminal unmyelinated axons.

K.H. Körtje: Are there differences in the reactivity of different types of glial cells? Does this mean that there are areas in your specimen with highly increased ATPase activity in glia and in neuronal profiles? Are these areas evenly distributed or can they be attributed to any defined substructure of the medial habenula?

Author: I think that there are differences, but these experiments are not sufficient to answer this question exactly. I did not find any answer to it in the literature, either. In this paper, my main purpose was to examine the distribution of ATPase activity around the nerve terminals.

T. Chiba: The specificity of the reaction products should be tested by control experiments. In this respect, inhibitory effects of increased amount of Na orthovanadate may be examined, although vanadate is not an inhibitor of just Ca-ATPase activity but is a characteristic inhibitor of all P-type ATPases.

Author: I did not use Na orthovanadate as a control experiment because, as described in the literature (45), it does not inhibit the ecto-ATPases, and the distribution of this ATPase activity undoubtedly showed "ecto" orientation.