FINE STRUCTURAL LOCALIZATION OF POTASSIUM-STIMULATED p-NITROPHENYLPHOSPHATASE ACTIVITY IN DENDRITES OF THE CEREBRAL CORTEX

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ABSTRACT

A histochemical technique for the demonstration of K⁺-p-nitrophenylphosphatase (K⁺-pNPPase) activity, a component of the Na⁺,K⁺-ATPase, has been applied at the fine structural level in the somatosensory cortex of the rat. Reaction product was consistently found in dendrites and in association with the cytoplasmic aspect of the dendritic plasmalemma. Reaction product often filled portions of the tubular smooth endoplasmic reticulum in these processes. The results of these studies are interpreted to indicate that enzymatic activity is associated with large- and small-diameter dendrites. No convincing evidence of high activity was found in glial profiles.

The importance of neurons and their dendrites in active transport of sodium and potassium ions in the cerebral cortex may be more significant than indicated by studies with isolated neurons and glia.

KEY WORDS p-nitrophenylphosphatase · Na⁺,K⁺-ATPase · dendrites · cerebral cortex · electron microscopy

An important advance in enzyme histochemistry has been the demonstration of K⁺-p-nitrophenylphosphatase (K⁺-pNPPase) activity, a component of the Na⁺,K⁺-ATPase (1, 4, 27) by the technique of Ernst (6, 7). This method is applicable for light and electron microscopy and has been utilized to localize K⁺-pNPPase activity in the secretory epithelium of the avian salt gland (6, 7, 9), in the endothelium of the rat cornea (16), in the lateral nasal gland of the desert iguana (5), and in cells of the renal distal tubule of the rat (8, 10). This technique has been validated utilizing avian salt gland (9), where [³H]ouabain, an established inhibitor of the Na⁺,K⁺-ATPase, binds to sites having the same distribution as the K⁺-pNPPase activity. Kyte (15) has also independently used immunocytochemical techniques to confirm that the basolateral plasma membrane of the distal renal tubule contains Na⁺,K⁺-ATPase-binding sites.

The application of the Ernst histochemical technique to localization of K⁺-pNPPase activity in cerebral cortex is especially important since there are conflicting biochemical data on whether neurons possess appreciable Na⁺,K⁺-ATPase activity (cf. reference 30 for review of this problem).

Our efforts to localize K⁺-pNPPase activity in the somatosensory cortex of the rat by light microscope histochemical methods led to the conclusion that much of the enzymatic activity resided in neuronal processes (29, 30). Although other structures did show activity, the more pronounced deposits of reaction product were associated with vertically oriented bundles of large structures ~3-6 μm in diameter. These appeared to arise in layer five of the cerebral cortex and arborize in...
more superficial layers. Since an equivalent arrangement of cortical dendritic processes has been described using light and electron microscope methods (see References 11, 12, 20, 31, and a review by Towe, 32), it was thought that the highly reactive, large-diameter, vertically oriented structures seen in our preparations were apical dendritic processes. This report deals with the identification of dendrites and associated sites of K⁺-pNPPase activity in the somatosensory cortex of the rat by electron microscopy.

MATERIALS AND METHODS
The animals used were adult male Sprague-Dawley rats. Anesthetized animals were perfused through their ascending aortas with chilled 3% formaldehyde plus 0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 20 min. After an additional 20 min, brains were removed and cortical areas 1, 2, and 3 (14) were dissected out. Blocks of cortical material were sectioned at 50 µm with a Vibratome.

Ernst's procedure (6, 7) was used to demonstrate K⁺-pNPPase activity in these sections as described in detail elsewhere (29, 30), except that the concentration of Pb(NO₃)₂ was reduced to 4 mM. This modification in the application of this technique was found to be critical to nervous tissue if preservation of morphology was to be obtained. In brief, the procedure was as follows: Vibratome slices were incubated in a medium containing 20 mM p-nitrophenylphosphate, 10 mM MgCl₂, 1.7 mM levamisole, 10 mM KC, 10 mM SrCl₂, and 100 mM Tris-HCl, pH 9, +-- 10 mM ouabain for 1 h at 30°C (29, 30). The slices were rinsed for 5 min in 100 mM Tris-HCl, pH 9, ± 10 mM ouabain for 1 h at 30°C (29, 30). The slices were then rinsed in 0.25% glutaraldehyde buffer, pH 7.4. After histochemical treatment, the tissue was postfixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, dehydrated with acetone, and embedded in Epon. Thin sections were obtained from blocks oriented with the cortical surface at the top and from blocks oriented so that sections were cut tangential to the cortical surface. Unstained thin sections and thin sections stained with uranyl acetate and lead citrate were examined with an electron microscope.

K⁺-pNPPase activity was determined in slices of somatosensory cortex from unfixed brain and from brain fixed as described above. The chemical assay utilized the same medium used in the histochemical localization of K⁺-pNPPase activity. This approach was described earlier (29).

RESULTS AND DISCUSSION
Early experience indicated that brief 5- to 10-min fixations of somatosensory cortex in 3% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, resulted in a 10-30% loss in K⁺-pNPPase activity (29). We can now report that longer, 40-min exposures to this fixative containing 0.25% glutaraldehyde result in an average additional loss of ~13% enzymatic activity. Although we have looked carefully, we have not been able to detect differences in the histochemical distribution of reaction product, that is, loss of reaction product from sites showing reactivity in preparations fixed for shorter times. Preservation of morphology was improved by the longer exposure to the fixative, as was expected.

Reaction product was not associated with every dendritic process seen in the electron microscope, but good examples of dendritic activity were found throughout the cortex. Profiles of large, reactive dendrites were observed in cross and longitudinal section (Figs. 1 and 2). Smaller-diameter dendrites also showed reactivity as illustrated in Figs. 3 and 4. In some cases, it was possible to demonstrate that reaction product was associated preferentially with the cytoplasmic aspect of the dendritic plasmalemma (Figs. 2 and 4). The reaction product at the plasmalemma often occurred in patches or clumps, and those portions of the membrane between the patches of reaction product were usually free of precipitate (Figs. 1–4). Reaction product sometimes filled all or portions of the small, vesicle-like structures comprising the smooth endoplasmic reticulum of these dendrites as seen in Fig. 1 and to better advantage in Fig. 2. K⁺-pNPPase activity was rarely found associated with mitochondrial membranes or with the neurotubules of these dendrites (Figs. 1–4). Reaction product was less consistently found in neuronal spine tips, axons, and perikarya. We were unable to find convincing examples of deposits of reaction product in glial profiles. Control sections incubated in media containing ouabain or without KC did not have precipitate deposited at membrane surfaces.

The identification of K⁺-pNPPase-generated reaction product in dendrites of the somatosensory cortex of the rat by electron microscope techniques confirms our earlier, tentative conclusion that K⁺-pNPPase activity resided in dendrites (29, 30). This has not been proven previously. With regard to other localizations of enzymatic activity, the tubular elements of the smooth-surfaced endoplasmic reticulum were often partially or completely filled with reaction product, and Na⁺,K⁺-ATPase activity has been localized in cell fractions thought to contain fragments of smooth endoplasmic reticulum (2, 17). Na⁺,K⁺-ATPase activity was not present in purified mitochondria isolated
Figure 1 Cross section of a large-diameter dendrite. $K^+$-pNPPase-generated reaction product is precipitated at the dendritic plasmalemma (arrows) and in some profiles of the smooth endoplasmic reticulum. Bar, 1.0 μm.

Figure 2 Longitudinal section of a large-diameter dendrite. $K^+$-pNPPase-generated reaction product can be seen precipitated at the cytoplasmic surface of the dendritic plasmalemma (small arrows) and filling or partially filling portions of the smooth endoplasmic reticulum (large arrows). Bar, 0.5 μm.

Figure 3 A medium-sized dendrite with $K^+$-pNPPase-generated reaction product at the plasmalemma (arrows). Bar, 0.5 μm.

Figure 4 A medium-sized dendrite with $K^+$-pNPPase-generated reaction product at the cytoplasmic aspect of the plasmalemma (small arrows) and filling elements of the smooth endoplasmic reticulum (large arrows). Bar, 0.5 μm.
from homogenates of rat brain (28), and reaction product was not found associated with these structures histochemically. The enzymatic activity localized in our studies was K+-dependent and could be inhibited by the cardiac glycoside, ouabain. Both of these features are characteristic of K+-pNPPase activity (1, 4, 27). Localization of the reaction product at the cytoplasmic aspect of the dendritic plasmalemma reported here would also be expected if the enzymatic activity was Na+,K+-ATPase related (1, 4, 27). The stimulation of activity by K+ along with ouabain inhibition of activity and the localization of reaction product on the cytoplasmic side of the dendritic plasmalemma led to the conclusion that Na+,K+-ATPase-related enzymatic activity had been localized in dendrites in the somatosensory cortex of the rat.

It is important to note that the results of studies of glial-rich and neuronal-rich fractions obtained from homogenates of whole brain are discrepant. Some investigators have found higher Na+,K+-ATPase activities in glial fractions than in neuronal fractions (18, 19), while others have not (13). Related to this, it has been generally accepted that the total volume of the cytoplasm of neuronal processes is greater, sometimes many times greater, than the volume of the perikaryon. However, illustrations of the “cells” obtained after cell isolation procedures have clearly shown the so-called neuronal-rich fraction to be composed of neuron cell bodies with the axons and dendrites shorn off (3, 18, 19, 21, 23). If a major portion of the neuronal Na+,K+-ATPase activity resided in dendrites, as our studies indicated, then it is probable that the proportion of neuronal to glial Na+,K+-ATPase activity has been underestimated and that the relative importance of glia in the transport of Na+ and K+ has been overestimated.

From a functional standpoint, Na+,K+-ATPase activity is directly proportional to ion flux (1), and the importance of K+-pNPPase activity to dendrite function may be related to the physiological role of dendrites in initiating membrane generator potentials for electrical conduction (22, 25). This warrants additional study, as do the possible changes in dendritic K+-pNPPase activity in altered physiological states. For example, there is evidence that Na+,K+-ATPase activity is decreased from normal levels in human epileptic foci (24). It is intriguing that there is also a decrease in the number of spines on apical dendrites of neurons in epileptic foci (26). Further study using this histochemical approach may permit examination of such possible interrelationships between enzymatic activity and morphological alteration.

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REFERENCES
1. BONTING, S. L. 1970. Sodium-potassium activated adenosinetriphosphatase and cation transport. In Membranes and Ion Transport. Vol. I. E. E. Bittar, editor. John Wiley & Sons, Inc., New York. 257-363.
2. BRADFORD, H. F., P. D. SWANSON, and D. B. GAMMACK. 1964. Constituents of a microsomal fraction from the mammalian brain: their solubilization, especially by detergents. Biochem. J. 92:247-254.
3. CHAO, S. W., and M. G. RUMSBY. 1977. Preparation of astrocytes, neurons and oligodendrocytes from the same rat brain. Brain Res. 124:347-351.
4. DART, J. L., and L. E. HOKEIN. 1974. The sodium-potassium adenosine-triphosphatase. Annu. Rev. Biochem. 43:327-356.
5. ELLIS, R. A., and C. C. GOERTEMULLER, JR. 1974. Cytological effects of salt-stress and localization of transport adenosine triphosphatase in the lateral nasal glands of the desert iguana Dipsosaurus dorsalis. Anat. Rec. 180:285-296.
6. ERNST, S. A. 1972. Transport adenosine triphosphatase cytochemistry. I. Biochemical characterization of a cytochemical medium for the ultrastructural localization of ouabain-sensitive, potassium-dependent phosphatase activity in the secretory epithelium of the avian salt gland. J. Histochem. Cytochem. 20:13-22.
7. ERNST, S. A. 1972. Transport adenosine triphosphatase cytochemistry. II. Cytotoxic characterization of ouabain-sensitive, potassium-dependent phosphatase activity in the secretory epithelium of the avian salt gland. J. Histochem. Cytochem. 20:23-38.
8. ERNST, S. A. 1975. Transport ATPase cytochemistry: ultrastructural localization of potassium-dependent and potassium-independent phosphatase activities in rat kidney cortex. J. Cell Biol. 66:586-608.
9. ERNST, S. A., and J. W. MILLS. 1977. Basolateral plasma membrane localization of ouabain-sensitive sodium transport sites in the secretory epithelium of the avian salt gland. J. Cell Biol. 75:74-94.

10. Firth, J. A. 1974. Problems of specificity in the use of a strontium capture technique for the cytochemical localization of ouabain-sensitive, potassium-dependent phosphatase in mammalian renal tissues. J. Histochem. Cytochem. 22:1163-1168.

11. Fleischhauer, K., and K. DETZER. 1975. Dendritic bundling in cerebral cortex. Adv. Neurol. 12:71-77.

12. Fleischhauer, K., H. Petsche, and W. Wittkowski. 1972. Vertical bundles of dendrites in neocortex. Z. Anat. Entwicklungsgesch. 136:213-223.

13. Hamberger, A., C. Blomstrand, and A. L. Lehninger. 1970. Comparative studies on mitochondria isolated from neuron-enriched and glia-enriched fractions of rabbit and beef brain. J. Cell Biol. 45:221-234.

14. Kreig, W. J. S. 1946. Connections of the cerebral cortex. I. The albino rat. A. Topography of the cortical areas. J. Comp. Neurol. 84:221-275.

15. Kyte, J. 1976. Immunoferritin determination of the distribution of (Na + + K +)ATPase over the plasma membranes of renal convoluted tubules. I. Distal segment. J. Cell Biol. 68:287-303.

16. Leukenberger, P. M., and A. B. Novikoff. 1974. Localization of transport adenosinetriphosphatase in rat cornes. J. Cell Biol. 66:721-731.

17. McIlwain, H. 1966. Biochemistry of the Central Nervous System. Little, Brown and Co., Boston. 250-252. 3rd edition.

18. Medzihradszky, F., P. S. Nandhasri, V. Idoiyaga-Varas, and O. Z. Sellinger. 1971. A comparison of the ATPase activity of the glial cell fraction and the neuronal perikaryal fraction isolated in bulk from rat cerebral cortex. J. Neurochem. 18:1599-1603.

19. Medzihradszky, F., O. Z. Sellinger, P. S. Nandhasri, and J. C. Santiago. 1974. Adenosine triphosphatase activity in glial cells and in neuronal perikarya of edematous rat brain. Brain Res. 67:133-139.

20. Peters, A., and T. M. Walsh. 1972. A study of the organization of apical dendrites in the somatic sensory cortex of the rat. J. Comp. Neurol. 144:253-268.

21. Poduslo, S. E., and G. M. McKhann. 1977. Maintenance of neurons isolated in bulk from rat brain: incorporation of radiolabeled substances. Brain Res. 132:107-120.

22. Purpura, D. P. 1967. Comparative physiology of dendrites. In The Neurosciences: A Study Program. G. C. Quarton, T. Melnechuk, and F. O. Schmitt editors. The Rockefeller University Press, New York. 372-393.

23. Raina, C. S., S. E. Poduslo, and W. T. Norton. 1971. The ultrastructure of purified preparations of neurons and glial cells. Brain Res. 27:11-24.

24. Rapport, R. L., II, A. B. Harris, P. N. Freel, and G. A. Ojemann. 1975. Human epileptic brain, Na,K-ATPase activity and phenytoin concentrations. Arch. Neurol. 33:549-554.

25. Rodolfo, L. 1975. Electroresponsive properties of dendrites in central neurons. Adv. Neurol. 12:1-13.

26. Scheibel, M. E., P. H. Crandall, and A. B. Scheibel. 1974. The hippocampal-dentate complex in temporal lobe epilepsy. Epilepsia. 15:55-80.

27. Skou, J. C. 1974. The (Na + + K +) activated enzyme system and its relationship to transport of sodium and potassium. Q. Rev. Biophys. 7:401-434.

28. Stahl, W. L. 1968. The subcellular distribution and nucleotide specificities of Na+,K+-stimulated adenosine triphosphatase and [3H]adenosine diphosphate-adenosine triphosphate exchange reactions in rat brain. J. Neurochem. 15:499-509.

29. Stahl, W. L., and S. H. Brodererson. 1976. Histochemical localization of potassium-stimulated p-nitrophenylphosphatase activity in the somatosensory cortex of the rat. J. Histochem. Cytochem. 24:731-739.

30. Stahl, W. L., and S. H. Brodererson. 1976. Localization of Na+,K+-ATPase in brain. Fed. Proc. 35:1260-1265.

31. Szentagothai, J. 1975. The 'module-concept' in cerebral cortex architecture. Brain Res. 95:475-496.

32. Towe, A. L. 1975. Notes on the hypothesis of columnar organization in somatosensory cerebral cortex. In Brain, Behavior and Evolution. Vol. 11. W. Riss, editor. S. Karger, Basel, Switzerland. 16-47.