Fluoride-Resistant Acid Phosphatase (FRAP)-Positive Primary Afferent Central Terminals in the Mouse Substantia Gelatinosa

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The ultrastructure of the central terminals of nociceptive primary afferent (CI terminals) neurons in the mouse substantia gelatinosa was studied by the cytochemical method using fluoride-resistant acid phosphatase (FRAP). Small roundish, slender, sinuous and large scalloped terminals showed FRAP reactivity. The FRAP reaction was seen as electron dense granular deposits between spherical synaptic vesicles and around the membrane of the synaptic vesicles. Especially, dark, small sinuous and large scalloped terminals showed intense FRAP reactivity. From the previous studies, these terminals were in

accord with the capsaicin-sensitive CI terminals. Furthermore, larger, roundish terminals with less packed spherical synaptic vesicles, many mitochondria and a few dense cored synaptic vesicles showed FRAP reactivity. Our recent results that CII terminals are also sensitive to capsaicin support the FRAP activity of the CII terminals in the present study. Thus, various types of FRAP-positive CI terminals and some CII terminals are considered to be central endings of the capsaicin-sensitive nociceptive primary afferents.

Key words: Fluoride-resistant acid phosphatase, Primary afferent neuron, Central terminal, Substantia gelatinosa, Mouse

I. Introduction

Central axons of nociceptive unmyelinated primary afferents are thought to terminate in the substantia gelatinosa (layer II) of the dorsal horn in mammals and centrally situated surrounded by many dendrites and a few axonal terminals, forming synaptic glomeruli [5, 29]. Type I synaptic glomeruli termed by Ribeiro-da-Silva and Coimbra (1982) have dark, large scalloped, central terminals (CI terminal) with closely packed spherical vesicles of variable diameter and few mitochondria, and are confined to lamina II in rats. At the early postnatal period, CI terminals are small dark, slightly sinuous with less packed synaptic vesicles and are non-glomerular [21]. During development, they generate the dark, large scalloped central glomerular endings [21]. The same type of CI terminals were demonstrated in the mouse substantia gelatinosa [6, 9]. Besides the typical, dark, large scalloped CI terminals, small round, slender or dark sinuous ter-

minals with closely packed or less packed clear synaptic vesicles were easily visualized, even in the adult mouse substantia gelatinosa. From the simple ultrastructural details, it is very difficult to determine whether these axonal terminals are of nociceptive primary afferent origin. After capsaicin treatment, CI terminals promptly degenerate and changed to shrunken profiles, so that determination of the original profiles of the degenerated terminals is not too easy [6, 20]. It is well established that fluoride-resistant acid phosphatase (FRAP) is specifically contained in the smaller primary afferent neurons and their peripheral and central terminals [14, 28]. Therefore, we used FRAP as a marker enzyme for the detection of small primary afferent central terminals in the mouse substantia gelatinosa.

II. Materials and Methods

For histochemical demonstration of FRAP, the method of Knyihár and Csillik (1981) was employed. Anesthetized (Nembutal, 50 mg/Kg) adult ddY mice were transcardially perfused with saline (50 ml), followed by a mixture of 4% glutaraldehyde and 3.2% paraform-

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Fig. 1. Several types of FRAP-reactive C terminals in the mouse substantia gelatinosa. FRAP-reactions are assessed by coarse dark granular or fine granular deposits (small arrows) in the axoplasm. $\times 51,000$. **a, b:** Small roundish reactive CI terminals packed with spherical synaptic vesicles. They make presynaptic contacts (asterisks) with surrounding dendrites (D). FRAP reactivities are seen between synaptic vesicles and around the synaptic membrane. A partial synaptic cleft (large arrows) show FRAP reactivity. Electron-dense mitochondria (M) are seen. **c:** A slender CI terminal showing fine granular FRAP reactivity (small arrows) among spherical synaptic vesicles. Synaptic cleft is also FRAP-reactive (large arrows). **d, e:** Small sinuous CI terminals showing intense FRAP reactivities. Surrounding dendrites (D) are FRAP-negative. **f:** A large scalloped, typical CI terminal showing FRAP-reactivity between packed spherical synaptic vesicles and around the membrane of the synaptic vesicles. At the lower side, the same type of FRAP-reaction is seen.

aldehyde in 0.2 M sodium cacodylate buffer, pH 7.2. The lumbar spinal cord was dissected out and placed in the same fixative for 2 hr at 4°C. Transverse sections at 40 μ m thickness were cut with a Vibratome (Lancer), and collected in 0.2 M sodium cacodylate buffer for at least 30 min. Then the sections were incubated in the mixture of 1.25% sodium- β -glycerophosphate and 0.2% $\text{Pb}(\text{NO}_3)_2$ in Tris-maleate buffer, pH 5.0, for 30 min at 37°C. It is established that FRAP is resistant to 0.2–0.4 mM sodium

fluoride, so an incubation medium without NaF was used. The sections were rinsed in Tris-maleate buffer 5 times and postfixed in 1.3% OsO_4 buffered with s-collidine for 1 hr at 4°C. S-collidine is used to buffer osmic acid and for the development of precipitate of the enzyme reaction [15]. Then sections were routinely dehydrated and embedded in Epon resin. Superficial dorsal horn, especially lamina II was trimmed under a binocular microscope, and they were coronally cut with an ultramicrotome, lightly stained

Fig. 2. Large round CII terminals with less packed clear synaptic vesicles and many mitochondria (M). $\times 51,000$. **a, b:** FRAP reactivities (small arrows) are seen between synaptic vesicles and around the membrane of the synaptic vesicles. Mitochondria (M) seems to show intense non-enzymatic precipitation. **c:** A normal CII terminal including many clear synaptic vesicles and mitochondria (M). Several dendrites (D) are postsynaptic to the CII terminal. An axonal terminal (A) is in contact with the CII terminal.

with uranyl acetate and lead citrate and examined in a Hitachi H-800 electron microscope.

III. Results

According to the criteria of Knyihár-Csillik and Csillik (1981) and Ribeiro-da-Silva *et al.* (1986), granular deposits of enzymatic reaction between the synaptic vesicles or over synaptic membrane and around the axolemma were cited as being due to FRAP reactivity in the axonal terminals. Various shaped C terminals, i.e., small round, slender, sinuous and dark, large scalloped terminals with closely packed spherical synaptic vesicles and few mitochondria showed FRAP reactivity (Fig. 1). Another type of larger roundish terminal with less packed synaptic vesicles and many mitochondria also showed FRAP reactivity (Fig. 2a, b). Most of these FRAP-positive terminals were surrounded by many dendrites and a few axonal endings, and formed synaptic glomeruli. The former is classified as TI-type synaptic glomeruli and the latter TII-type. The central FRAP-positive terminals are called CI-terminals and CII-terminals, respectively. For comparison of the FRAP-reactive CII terminals, a normal CII terminal is presented in Fig. 2c. Nonglomerular FRAP-positive terminals were also seen in the substantia gelatinosa (data not shown). Mitochondria showed electron-dense profiles in FRAP-positive and -negative C terminals and dendrites (Figs. 1, 2).

IV. Discussion

There are many studies in which FRAP is used as a marker enzyme of the primary afferent neurons [1-3, 11, 12, 15-18, 24, 25]. FRAP has been proved to be synthesized in the smaller primary afferent dorsal root ganglion neurons and transported to their central and peripheral terminals by analyzing sections of dorsal root and sciatic nerve [12] and ligation of the peripheral nerve [2, 17]. For example, after neurectomy (see review 14) and capsaicin treatment [11], the FRAP reaction disappears in the superficial dorsal horn ipsilateral to the lesion and in the capsaicin-treated dorsal horn, respectively.

In the neonatal rat substantia gelatinosa, dark, small round, sinuous terminals sensitive to capsaicin were seen [21]. They develop into electron dense, large scalloped CI terminals, therefore immature small terminals diminish in number with growth of the animal [21]. However, small round, slender, sinuous terminals with or without packed clear synaptic vesicles are abundant in the adult mice (unpublished observation). Capsaicin rapidly degenerates C terminals, resulting in the high electron-dense shrunken profiles. The identification of their original figures of C terminals sensitive to capsaicin is very difficult to ascertain [7, 20]. Thus, FRAP reactivity was used for the identification of nociceptive primary afferent C terminals.

As FRAP is a nonlysosomal enzyme scattered

throughout the axoplasm [13, 15, 24], FRAP reactivity was determined as electron-dense deposits among synaptic vesicles or over synaptic membranes and around the axolemma [24]. The mitochondria exhibited FRAP-like reactivity in FRAP positive and non positive terminals and dendrites. As the mitochondria are unstained with FRAP [11], the reason for the mitochondrial FRAP-like reaction is not known. After peripheral nerve crush, degenerated axonal mitochondria exhibited precipitation of lead phosphate [10]. Several types of axon terminals showed FRAP reactivity: one type consists of small round, slender, dark sinuous and large scalloped terminals with packed clear synaptic vesicles, and the other consists of larger roundish terminals with less packed clear synaptic vesicles and many mitochondria. The former corresponds to the dark sinusoid axon terminals (DSA) [13-16] or CI terminals [22], and the latter corresponds to CII terminals [22]. The CI terminals are believed to be central processes of unmyelinated nerve fibers and capsaicin-sensitive [19, 23]. On the contrary, the CII-terminals are believed to be central processes of thin myelinated nerve fibers and capsaicin insensitive [23]. It was shown that most of the CI terminals (78%) are FRAP positive, whereas none of the CII terminals are FRAP positive [24], but in the present study both C terminals showed FRAP reactivity. The difference of FRAP reactivity in the CII terminals may indicate different origins of this type of terminal in the rat and mouse (species difference). From previous studies FRAP reactivity is localized in the substantia gelatinosa (layer II) of the rat dorsal horn [1-4, 8, 11-17, 24, 25, 27]. Nociceptive primary afferent central terminals terminate in the substantia gelatinosa [5, 29]. Jancsó *et al.* (1975) reported that, after subcutaneous injection of capsaicin, FRAP activity in the terminal portion of thin dorsal root axons disappeared in the substantia gelatinosa. In addition, FRAP activity is completely abolished in the substantia gelatinosa by capsaicin. From these results, they suggest that FRAP is related to the processing of nociceptive impulses. The present results showed that FRAP-positive terminals are synonymous with the various types of CI terminals sensitive to capsaicin [6-8]. Further, FRAP-reactive CII-terminals also support the existence of capsaicin-sensitive CII-terminals [6, 8].

V. References

1. Coimbra, A., Sodr-Borges, B. P. and Magalhes, M. M.: The substantia gelatinosa Rolandi of the rat. Fine structure, cytochemistry (acid phosphatase) and changes after dorsal root section. *J. Neurocytol.* 3; 199-217, 1974.
2. Devor, M. and Claman, D.: Mapping and plasticity of acid phosphatase afferents in the rat dorsal horn. *Brain Res.* 190; 17-28, 1980.
3. Ferencsik, M., Garay, K., Mihly, A. and Csillik, B.: Cobalt-complex ATP enhanced regeneration in the dorsal horn of the rat spinal cord. *Exp. Brain Res.* 76; 409-416, 1989.
4. Fitzgerald, M. and Vrbov, G.: Plasticity of acid phosphatase (FRAP) afferent terminal fields and of dorsal horn cell growth in

- the neonatal rat. *J. Comp. Neurol.* 240; 414–422, 1985.
5. Fyffe, R. E. W.: Laminar organization of primary afferent termination in the mammalian spinal cord. In "Sensory Neurons. Diversity, Development, and Plasticity", ed. by S. A. Scott, Oxford University Press, New York, 1992, pp. 131–139.
 6. Hiura, A. and Ishizuka, H.: Early morphological changes of primary afferent neurons and their processes in newborn mice after treatment with capsaicin. *Exp. Brain Res.* 101; 203–215, 1994.
 7. Hiura, A. and Ishizuka, H.: Central terminals of capsaicin-sensitive primary afferent make synaptic contacts with neuronal soma in the mouse substantia gelatinosa. *Experientia* 51; 551–555, 1995.
 8. Hiura, A., Villalobos, E. L. and Ishizuka, H.: The action of capsaicin on primary afferent central terminals in the superficial dorsal horn of newborn mice. *Arch. Histol. Cytol.* 53; 455–466, 1990.
 9. Hiura, A., Ishizuka, H. and Villalobos, E. L.: GABAergic neurons in the mouse superficial dorsal horn with special emphasis on their relation to primary afferent central terminals. *Arch. Histol. Cytol.* 54; 195–206, 1991.
 10. Holtzman, E. and Novikoff, A. B.: Lysosomes in the rat sciatic nerve following crush. *J. Cell Biol.* 27; 651–669, 1965.
 11. Jancsó, G. and Knyihár, E.: Functional linkage between nociception and fluoride-resistant acid phosphatase activity in the Rolando substance. *Neurobiology* 5; 42–43, 1975.
 12. Knyihár, E.: Fluoride-resistant acid phosphatase system of nociceptive dorsal root afferents. *Experientia* 27; 1205–1207, 1971.
 13. Knyihár, E. and Gerebtzoff, M. A.: Extra-lysosomal localization of acid phosphatase in the spinal cord of the rat. *Exp. Brain Res.* 18; 383–395, 1973.
 14. Knyihár, E. and Csillik, B.: Effect of peripheral axotomy on the fine structure and histochemistry of the Rolando substance: degenerative atrophy of central processes of pseudounipolar cells. *Exp. Brain Res.* 26; 73–87, 1976.
 15. Knyihár-Csillik, E. and Csillik, B.: FRAP: Histochemistry of the primary nociceptive neuron. *Prog. Histochem. Cytochem.* 14; 1–137, 1981.
 16. Knyihár, E., László, I. and Tornóyos, S.: Fine structure and fluoride resistant acid phosphatase activity of electron dense sinusoid terminals in the substantia gelatinosa Rolandi of the rat after dorsal root transection. *Exp. Brain Res.* 19; 529–544, 1974.
 17. McMahon, S. B.: The localization of fluoride-resistant acid phosphatase (FRAP) in the pelvic nerves and sacral spinal cord of rats. *Neurosci. Lett.* 64; 305–310, 1986.
 18. McNeill, D. L. and Hulsebosch, C. E.: Intraspinal sprouting of rat primary afferents after deafferentiation. *Neurosci. Lett.* 81; 57–62, 1987.
 19. Palermo, N. N., Brown, H. K. and Smith, D. L.: Selective neurotoxic action of capsaicin on glomerular C-type terminals in rat substantia gelatinosa. *Brain Res.* 208; 506–510, 1981.
 20. Pignatelli, D., Coimbra, A. and Zieglgänsberger, W.: Identification of C fiber primary afferent endings in the substantia gelatinosa of the newborn rat: an electron-microscopic study. In "Fine afferent nerve fibers and pain", ed. by R. F. Schmidt, H.-G. Schaible and C. Vahle-Hinz, VCH Publishers, Weinheim, 1987, pp. 69–75.
 21. Pignatelli, D., Ribeiro-da-Silva, A. and Coimbra, A.: Postnatal maturation of primary afferent terminations in the substantia gelatinosa of the rat spinal cord. an electron microscopic study. *Brain Res.* 491; 33–44, 1989.
 22. Ribeiro-da-Silva, A. and Coimbra, A.: Two types of synaptic glomeruli and their distribution in laminae I–III of the rat spinal cord. *J. Comp. Neurol.* 209; 176–186, 1982.
 23. Ribeiro-da-Silva, A. and Coimbra, A.: Capsaicin causes selective damage to type I synaptic glomeruli in rat substantia gelatinosa. *Brain Res.* 290; 380–383, 1984.
 24. Ribeiro-da-Silva, A., Castro-Lopes, J. M. and Coimbra, A.: Distribution of glomeruli with fluoride-resistant acid phosphatase (FRAP)-containing terminals in the substantia gelatinosa of the rat. *Brain Res.* 377; 323–329, 1986.
 25. Shehab, S. A. S. and Atkinson, M. E.: Vasoactive intestinal polypeptide increases in areas of the dorsal horn of the spinal cord from which other neuropeptides are depleted following peripheral axotomy. *Exp. Brain Res.* 62; 422–430, 1986.
 26. Silverman, J. D. and Kruger, L.: Acid phosphatase as a selective marker for a class of small sensory ganglion cells in several mammals: spinal cord distribution, histochemical properties, and relation to fluoride-resistant acid phosphatase (FRAP) of rodents. *Somatosens. Res.* 5; 219–246, 1988.
 27. Szabó, B. K., Török, A., Knyihár-Csillik, E. and Csillik, B.: Mapping the distribution of thiamine monophosphatase, fluoride-resistant acid phosphatase, and substance P in the spinal cord with a personal computer compatible program. *Acta. Histochem.* 87; 123–129, 1989.
 28. Willis, W. D. Jr. and Coggeshall, R. E.: Dorsal root ganglion cells and their processes. In "Sensory Mechanisms of the Spinal Cord", ed. by W. D. Willis and R. E. Coggeshall, Plenum Press, New York, 1991, pp. 47–78.
 29. Willis, W. D. Jr. and Coggeshall, R. E.: Structure of the dorsal horn. In "Sensory Mechanisms of the Spinal Cord", ed. by W. D. Willis Jr. and R. E. Coggeshall, Plenum Press, New York, 1991, pp. 79–151.