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Mini Review

Fluorescent AM1-43 and FM1-43 probes for dental sensory nerves and cells: Their labeling mechanisms and applications

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Summary This mini-review first introduces the fluorescent styryl dyes, AM1-43 and FM1-43, which stain a variety of sensory cells and tissues including sensory nerve fibers. Second, a photoconversion method for ultrastructural localization of the dyes within cells is described as an example of an applied technique of these fluorescent dyes. Third, the following labeling mechanisms of FM1-43, AM1-43 and related dyes are introduced: (1) internalization of the dyes via endocytosis, (2) direct dye internalization via cation channels in the plasma membrane, (3) labeling by membrane insertion of the dye molecules and extensive membrane accumulation in cell membranes such as myelin of nerves. Fourth, the application of AM1-43 as a tool for observation of the innervation of periodontal ligaments and dental pulp is introduced and a possible candidate dye-permeable cation channel protein is suggested. Fifth, several topics which should be focused on in future studies are raised. Accumulation of these dye applications may shed new light on further understanding of dental tissue formation and its maintenance.

Introduction

The dye molecule FM1-43 was first introduced by Betz et al. [1] as an activity-dependent endocytosis marker. In addition to being endocytosed, it has been suggested that FM1-43 passes through the mechanosensitive cation channel of lateral line hair cells [2]. FM1-43 and the similar but longer wave length dye FM4-64 are applicable to FITC and rhodamine optics, respectively [3]. These dyes are supplied by Molecular Probes, although equivalent dyes such as SynaptoGreen C4 and SynaptoRed C2 are available from Biotium. A fixable analogue of FM1-43, AM1-43, is also available from Biotium, and FM1-43FX and FM4-64FX are provided by Molecular Probes.

A variety of cells and tissues are labeled with FM1-43 and AM1-43

FM1-43 and related fluorescent dyes have been reported to label a motor nerve terminal [1,4], Drosophila neuromuscular junctions [5], pituitary lactotroph granules [6], intestinal brush borders [7], cultured hippocampal neurons [8], photoreceptor cells of the retina [9], and plant cells [10] as results of endocytosis. FM1-43 and related fluorescent dyes have also

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been reported to label hair cells of lateral line organs [2,11—20] and the inner ear [19,21—29]. Other sensory cells and nerves are also labeled with FM1-43, AM1-43 and related dyes: vibrissal follicles, palatal rugae, enteric neurons, nasal mucosa, Merkel cells, muscle spindles, cultured astrocytes, sensory neurons of trigeminal and dorsal root ganglia, sensory receptors of visceral pleura, and cough receptors of vagal sensory neurons [22,30—40].

Eimer's organ of the mole is an example of a complex sensory organ that can be labeled with AM1-43. This organ is sensitive to mechanosensory stimuli and consists of free nerve endings, Merkel cells and laminated corpuscles, all of which can be labeled with AM1-43 [41,42]. In addition, since some groups of the free nerve endings are immunohistochemically positive for substance P, at least some of these nerve fibers may play a nociceptive role. Interestingly, free nerve endings labeled with AM1-43 are often observed to reach the epidermis and to terminate at the stratum corneum, whereas similar nerve endings labeled with the anti-neurofilament protein NF-200 antibody terminate below the stratum corneum. The AM1-43-labeled nerve endings display ghostlike profiles in the stratum corneum when examined by electron microscopy, and appear to be degraded [42]. This observation suggests that remnants of degraded, but not living, nerve fibers can still be labeled by AM1-43, but not by anti-neurofilament proteins.

Photoconversion method for electron microscopic analysis of fluorescent labeled cells

Fluorescent dyes can be localized in cells by electron microscopy by using the photoconversion method. This method involves the conversion of fluorescent dyes to the electron-dense diaminobenzidine (DAB). Since Maranto [43] first showed photoconverted DAB products in a Lucifer yellow-labeled neuron, there have been several other reports which used this method employing a variety of fluorescent dyes [44—46]. FM1-43 and related dyes are also photoconverted in a variety of tissues and cells such as lateral line hair cells [2], frog motor terminals [47], hippocampal neurons [48,49], human T cells [50], pituitary lactotroph granules [51], PC12 cells [52], neurons of dorsal root ganglia [53] and Drosophila motor neurons [54]. Thus, photoconversion of FM1-43 or AM1-43 fluorescence is a powerful tool for the high resolution localization of labeled cellular structures.

Cellular labeling mechanisms of FM1-43 and AM1-43: endocytotic labeling and labeling through sensory channels

Introduction to the mechanisms of cell-dye labeling

Both FM1-43 and AM1-43 have a lipophilic tail and a highly hydrophilic, cationic head group. This means that, when the dyes are administered into the extracellular space, the dye molecule is inserted into the outer leaflet of the plasma membrane but cannot pass through the membrane because of its charged head group (Fig. 1) [1]. These dyes are virtually nonfluorescent in aqueous solution. However, the dyes are intensely fluorescent at the point of insertion of the lipophilic tail into the membrane. The dyes incorporated into the membrane continue to be highly fluorescent when taken up by endocytosis (Fig. 1A). These dyes can also enter cells by permeation through nonselective cation channels, following which they fluoresce within the cells possibly by incorporation into intracellular membrane organelles (Fig. 1B). Plasma membrane-rich structures such as myelin of nerve fibers provide many opportunities for lipophilic tail insertion of FM1-43 and AM1-43, which results in moderate fluorescence (Fig. 1C) [1,34]. It has also been suggested that FM dyes can label cells via uptake into the cell through a sodium pump. [40].

Endocytotic labeling

Since the pioneering work by Betz et al. [1] there have been a number of reports of FMI-43 labeling of cells via endocytosis of labeled membranes including labeling of endocytotic recycling synaptic vesicles of motor nerve fibers and endocytotic labeling of nerve fibers and terminals, lateral line hair cells, retinal photoreceptor cells, and even plant cells by FM1-43 and related dyes [2—4,6,8,9,55]. A review has also been published on FM1-43 labeling of Drosophila neuromuscular junctions via an endocytotic pathway [5].

Labeling through sensory cation channels

It is likely that fluorescent dyes can enter into cells via permeation of the mechanotransduction channels of the hair cells of the inner ear and lateral line organs [2,22]. Thus, FM1-43 is known to be a permeant blocker of the mechanotransduction channel of mouse cochlear hair cells [21] and of dorsal root ganglion neurons [56]. Meyers et al. [22] demonstrated that FM1-43 can pass through two different cation channels; the vanilloid receptor, TRPV1 and the purinergic receptor, $P2X_2$ in HEK293T cells that were transfected with the corresponding genes. TRPV1 is known to be expressed in the sensory neuron and to be important in the pain sensation [57,58]. There is increasing evidence to support the possibility that hair cells can be labeled with FM1-43, AM1-43 and related styryl dyes via permeation of mechano-electrical transduction channels [13—20,23,25—28]. On the other hand, other reports have suggested that hair cells are labeled with FM1-43 via the endocytotic pathway [24,59,60]. Since lateral line hair cells can show limited endocytosis of FM1-43 at the apical end [2], these reports have confirmed the endocytotic pathway as one mechanism for FM1-43 internalization. However, FM1-43 internalization via direct permeation through cation channels may also occur in diverse hair cells of the lateral line and the inner ear as well as in other cell types.

Although astrocytes are not sensory neurons, the FM1-43-related dye FM4-64, enters a store-operated calcium channel of cultured astrocytes [37]. Karashima et al. [61] reported on the other channel of transient receptor potential (TRP) family that TRPA1-transfected Chinese hamster ovary (CHO) cells are labeled with FM1-43 following stimulation with a TRPA1 agonist. TRPA1 is also expressed in the sensory neurons [25,61,62]. Therefore, at least FM1-43 can pass

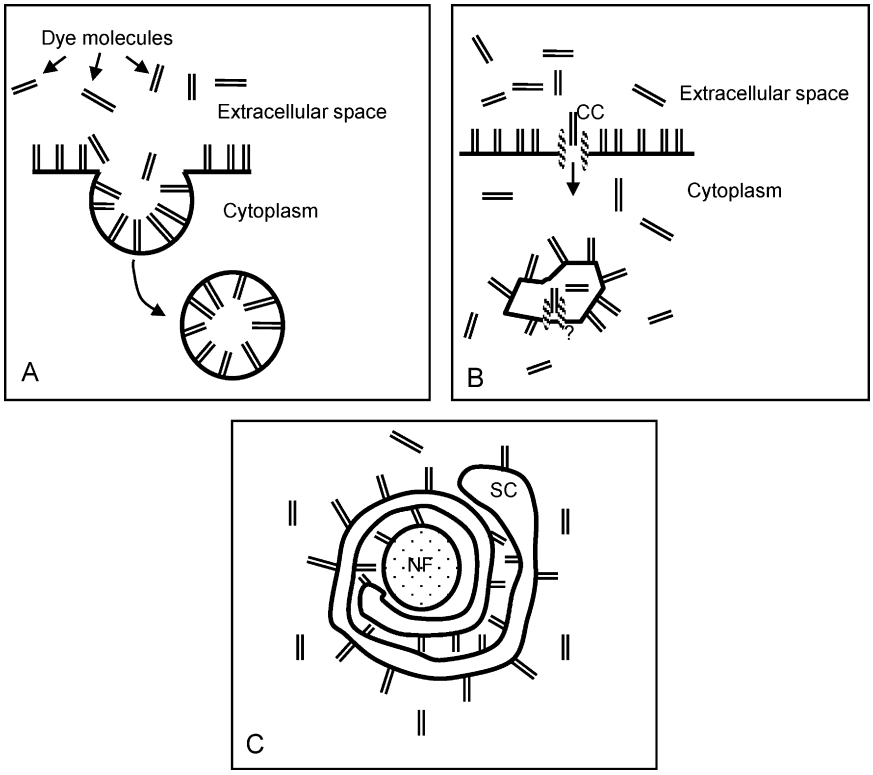

Figure 1 The three different cellular labeling mechanisms of FM1-43 and AM1-43. Intracellular dye fluorescence results from: (A) insertion of dye molecules into the plasma membrane and endocytosis of dye-containing membrane lacunae and (B) direct passage of dye-molecules through cation channels in the plasma membrane and possible incorporation into intracellular membrane organelles (question mark). (C) Dye-insertion into the plasma membrane of the myelin sheath of a Schwann cell (SC) around a nerve fiber (NF) also results in moderate dye fluorescence.

through the mechanosensitive cation channel of hair cells [2,21,22], as well as the store-operated calcium channel [37], TRPV1 [22], $P2X_2$ [22], and TRPA1 [61].

The diameter of FM1-43 and the pore size of the cation channel are important parameters that regulate the channel permeability of FM1-43. It has been shown that small organic cations with diameters up to ~0.8 nm can pass through the hair cell transducer channel. Since the triethylammonium end group of FM1-43 is 0.7 nm in size, FM1-43 should therefore be able to pass through this transducer channel [21]. Although the molecular weight of FMI-43 is 452, its long, thin shape is considered to facilitate the passage of FMI-43 through the channel. Furthermore, FM1-43 is a divalent cation and this property would be expected to provide a driving force for passage through the channel [22]. Karashima et al. [61] has reported that the diameter of FM1-43 is 10.5 Å and that channel pore sizes of TRPV1 and TRPA1 are 10.1 Å and at least 11 Å, respectively. After stimulation of these channels by agonists such as capsaicin or mustard oil, the channel pore is reported to be dilated and to show dynamic alterations in ion permeability [57,58,61,62]. Even TRPV2, TRPV3 and TRPV4 have been reported to facilitate the uptake of organic cationic dyes such as Yo-Pro after agonist stimulation [63].

FM1-43 also passes through another nonselective cation channel, $P2X_2$ [22]. P2X receptors are extracellularly activated, ATP-gated ion channels that mediate the rapid nonselective passage of cations (Na^+, K^+, Ca^{2+}) across the cell membrane, which results in an increase in intracellular Ca^{2+} and in depolarization [64]. P2X antagonists have been reported to inhibit AM1-43 entry into hair cells [65]. P2X receptors have also been reported to mediate pain sensation and hair cell mechanosensitization [65—67].

The two labeling mechanisms of FM1-43 and AM1-43 explain their induced fluorescence of nerves and sensory organs

In summary, the above data show that FM1-43 and AM1-43 can label cells in two ways; by mechanotransduction via ion channel membrane permeability and by endocytosis that is followed by exocytosis. These two pathways underlie the mechanism by which these dyes label nerves and sensory organs.

Other labeling mechanisms

Betz et al. [1] has reported that FM1-43 stains myelin of nerve fibers in a nerve activity-independent fashion resulting in a greenish fluorescence. The color of FM1-43 has been reported to be related to the concentration of the dye in secreted lactotroph granules at a high dye concentration the granule is red in color and at a low concentration it is green in color [6]. In periodontal ligaments, myelin sheaths of sensory nerve fibers are moderately labeled with AM1-43 that displays a

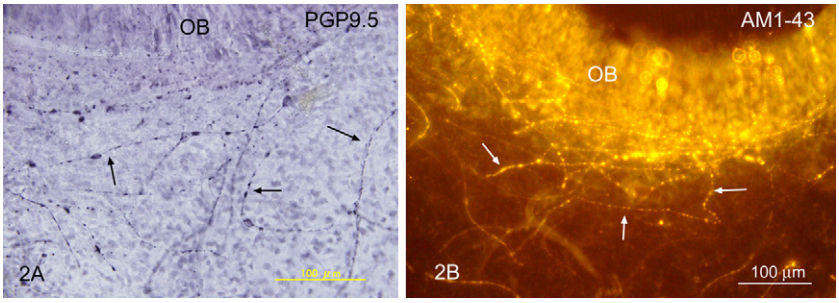

Figure 2 General neuronal marker PGP9.5 immunostaining and AM1-43 staining of sensory nerve fibers. Nerve fibers in the first molar pulp in the mandible of a 27-day-old rat were immunohistochemically stained with specific antibodies against PGP9.5 (A) or labeled with AM1-43 (B). The sub-odontoblastic plexus of sensory nerve fibers in the pulp area underneath the odontoblast layer (OB) is shown. AM1-43 was injected subcutaneously at the back of the animal. One day after the injection the animal was sacrificed, and the dissected mandible was fixed, demineralized and observed by fluorescence microscopy (B). PGP9.5 was analyzed by light microscopy (A) after immunohistochemical staining of cryosections. Anti-PGP9.5 and AM1-43 stained sensory nerve fibers are densely and brightly stained, respectively (arrows). Bars = 100 μm.

greenish fluorescence [34]. Thus, dye-insertion into the plasma membrane of the myelin sheath of a Schwann cell around a nerve fiber results in moderate dye fluorescence (Fig. 1C).

Another mechanism of FM dye labeling via a membrane sodium pump has been suggested for the cough receptors of airway sensory nerves which are brightly fluorescent after intravital loading of the FM dye [40].

Labeling of dental pulp and periodontal ligaments of mouse and rats with AM1-43

Mouse dental pulp and periodontal ligaments

The first report that AM1-43 labels the sensory nerve fibers of mouse dental pulp and periodontal ligaments was by Nishikawa [33]. In that study, adult mice (30—35 g) were subcutaneously injected with a small amount of AM1-43 (2 μg). One to 3 days after AM1-43 injection, the animals were fixed with paraformaldehyde (PFA) or with PFA plus glutaraldehyde, and were then demineralized with a solution of EDTA. The periodontal ligaments of molars and incisors contained large bright mechanoreceptors which may include Ruffini corpuscles. In another study of incisor periodontal ligaments, fine nerve fibers different from large Ruffini corpuscles were labeled with AM1-43, which were probably nociceptive, free nerve endings [68]. In the dental pulp, AM1-43-positive sensory nerve fibers are abundant in the molar pulp but are fewer in number in the incisor pulp. Moreover, in the molar pulp bright AM1-43-positive sensory nerve fibers pass through the odontoblast layer and even penetrate into dentin up to a depth of around 100 μm. Co-staining of AM1-43 with the general neuronal marker PGP9.5 confirmed that the AM1-43-labeled fiber is either a nerve fiber or a nerve terminal that penetrates dental pulp or periodontal ligaments. AM1-43-positive nerve fibers as well as AM1-43-positive Merkel cells are abundant in gingiva. Since most sensory fibers originate from the trigeminal ganglion, neuronal cell bodies of the trigeminal ganglion are brightly labeled with AM1-43. In addition, ameloblasts are also moderately labeled with AM1-43. The labeling intensity of AMI-43 is stronger in maturation ameloblasts than in secretion ameloblasts. The labeled part of the cell is the cytoplasm that is close to the nucleus. Since it is unlikely that ameloblasts express membrane permeable channels, this labeling may reflect the endocytotic activity of ameloblasts. Odontoblasts in the dental pulp of mice and rats are sometimes labeled with AM1-43 moderately (Fig. 2B). Thus, further study is needed to elucidate the labeling mechanisms of these non-neural cells.

AM1-43 labeling reveals differential pulp innervation in molars and incisors during tooth development of rats

AM1-43 labeling has revealed innervation of the dental pulp of the first molar and incisor in 3-, 7-, 15-, 27- and 41-day-old rats [69]. Identification and distribution of nerve fibers in the dental pulp has been widely studied using autoradiography and neuron-specific antibodies [70,71]. However, AM1-43 labeling is a more powerful tool for detection of sensory nerve fibers in the dental pulp since it specifically labels these nerve fibers and does not label autonomic nerve fibers in the submandibular gland. For example, AM1-43 labeling has revealed innervation of the dental pulp of the first molar and incisor in 3-, 7-, 15-, 27- and 41-day-old rats [69]. In this study, AM1-43-positive sensory fibers were absent in the pulp of 3-day-old rat, but first noted in the pulp of the first molar and incisor in the 7-day-old rat. This study further showed that, although sensory fibers are sparsely distributed in the pulp and do not reach the odontoblast layer in the incisors of rats between the ages of 7 and 41 days old, the sensory fibers do penetrate into the odontoblast layer and even into dentin up to a depth of 100 μm in the 15-day-old first molar of the rat. In the first molar pulp of a 27-day-old rat there was a well developed plexus of sensory fibers underneath the odontoblast layer. The neuronal nature of these fibers was immunohistochemically confirmed using the general neuronal marker, PGP9.5 (Fig. 2). In contrast, in

the incisor pulp of 15-day-old and older animals, only a few AM1-43-positive fibers were visible along blood vessels. This AM1-43-labeling study therefore indicated different innervation in the pulp between molars and continuously erupting rodent incisors.

Co-staining of TRPV2 and AM1-43 in sensory nerve fibers of rat molar pulp and trigeminal ganglion cells

It remains to be clarified why sensory nerve fibers are labeled in an activity-independent manner with AM1-43. Although it is possible that these fibers may be labeled via endocytic mechanisms, direct internalization of AM1-43 into nerve fibers via transient receptor potential (TRP) ion channels is more likely. Meyers et al. [22] have reported that TRPV1 that is activated by noxious heat and capsaicin, is permeable to FM1-43. Therefore, TRPV1 or its relatives such as TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1 are also possible candidates for mediating AM1-43 cell-labeling [63,72,73]. In an immunocytochemical study, Nishikawa [35] reported that some of the AM1-43-labeled sensory nerve fibers in rat molar dental pulp co-stained with the TRPV2 channel protein. Furthermore, some trigeminal ganglion cells, a few of which innervate the dental pulp, were double labeled with AM1-43 and anti-TRPV2. These results suggest that the TRPV2 channels of some sensory nerve fibers may be responsible for their bright labeling with AM1-43, by allowing the dye to pass through the channels.

When a branch of the trigeminal nerve was ligated in neonatal mice, followed by AM1-43 administration at the ipsilateral whisker follicle region distal to the ligated portion, few neurons were labeled in the trigeminal ganglion after 24 h [22]. On the other hand, contralateral trigeminal ganglion neurons without ligation were labeled by AM1-43. Thus, AM1-43 enters the sensory terminal of nerve fibers and is then transported to the cell bodies [22]. In the case of dental pulp nerve fibers, TRPV2 is a candidate TRP channel for AM1-43 permeation.

Further points to be clarified in the labeling of dental tissues with FM1-43 and AM1-43

Four points regarding the use of FMI-43 and AM1-43 that should be explored in future studies are raised below.

1. Comparative studies of AM1-43 staining of dental tissues in different mammalian species, in particular the staining of diverse sensory corpuscles such as Ruffini corpuscles in periodontal ligaments.
2. Further examination of AM1-43 labeling mechanisms in ameloblasts, especially in maturation ameloblasts, since they show only moderate cytoplasmic labeling and are not expected to express membrane-permeable channels [33].
3. Ultrastructural localization of AM1-43-labeled structures in dental pulp and periodontal ligaments using the photoconversion method, in order to elucidate the pathway of AM1-43 internalization into the cell.
4. Identification of candidate protein cation channels other than TRPV2 in dental tissues.

Conflict of interest statement

The author declares no conflict of interest.

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