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P. Dubový · J. Bednářová

The extracellular matrix of rat Pacinian corpuscles: an analysis of its fine structure

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Abstract The Pacinian corpuscle consists of a sensory axon terminal that is enveloped by two different structures, the inner core and the capsule. Since proteoglycans are extremely water soluble and are extracted by conventional methods for electron microscopy, the current picture of the structural composition of the extracellular matrix in the inner core and the capsule of the Pacinian corpuscle is incomplete. To study the structural composition of the extracellular matrix of the Pacinian corpuscles, cationic dyes (ruthenium red, alcian blue, acridine orange) and tannic acid were applied simultaneously with the aldehyde fixation. The interosseal Pacinian corpuscles of the rat were fixed either in 2% formaldehyde and 1.5% glutaraldehyde, with the addition of one of these cationic dyes or, in Zamboni's fixative, with tannic acid added. The cationic dyes and tannic acid revealed a different structural pattern of proteoglycans in the extracellular matrix in the inner core and in the capsule of the rat Pacinian corpuscles. The inner core surrounding the sensory axon terminal is a compartment containing proteoglycans that were distributed not only in the extracellular matrix but also in the cytoplasm of the lamellae. In addition, this excitable domain was separated from the capsular fluid by a thick layer of proteoglycans on its surface. An enlarged interlamellar space of the capsule contained large amounts of proteoglycans that were removed by digestion with chondroitinase-ABC. Ruthenium red and alcian blue provided only electron dense granules, probably corresponding to collapsed monomeric proteoglycan molecules. Acridine orange and tannic acid preserved proteoglycans very well and made it possible to visualize them as "bottlebrush" structures in the electron microscope. These results show that the inner core and the capsule of rat Pacinian corpuscles have different structural patterns of proteoglycans, which are probably involved in different functions.

P. Dubový (✉) · J. Bednářová
Department of Anatomy, Division of Neuroanatomy,
School of Medicine, Masaryk University, Brno, Kamenice 3,
CZ-625 00 Brno, Czech Republic
Tel.: +420-5-47121222
Fax: +420-5-43211225

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Introduction

The Pacinian corpuscle is a unique type of encapsulated sensory nerve ending with a typical oval-shaped onion-like structure. The corpuscle consists of three basic structural components: sensory terminal, inner core, and capsule. A cylindrical sensory axon terminal is directed parallel to the long axis of the corpuscle. The axon terminal, which appears as oval in cross section, is enveloped by two systems of densely packed hemilamellae with radial clefts between them – the inner core. The inner core is formed by cytoplasmic processes of the specialized Schwann cells, the bodies of which are situated at the outer margin of the inner core (Pease and Quilliam 1957; Poláček and Mazanec 1966; Munger and Idé 1988).

The inner core lamellae are separated from each other by very narrow spaces filled with amorphous ground matrix containing fine filamentous material and collagen fibrils with a diameter of about 15 nm (reviewed Munger et al. 1988; Bell et al. 1994). The interlamellar extracellular matrix (ECM) is continuous with that filling the radial clefts present at both ends of the Y-axis of the oval axon terminal. The cleft space contains amorphous matrix with collagen fibrils 30–40 nm in diameter, and elastic fibers of moderate opacity (Munger et al. 1988). Conventional electron microscopy has revealed that the basal lamina is present only on the outermost inner core lamellae (Chouchkov 1971; Idé and Hayashi 1987).

The unit of the sensory terminal-inner core is separated from surrounding tissue by a multilamellar complex derived from fibroblasts. The lamellae are arranged concentrically and separated from each other by a relatively wide space containing a fluid with scattered collagen fibrils. In contrast, the outermost 5–7 lamellae have narrow interlamellar spaces filled by densely packed collagen fibrils. Both external and internal surfaces of all la-

mellae are invested by the basal lamina (Halata 1975; Zelená 1994). Since the lamellae have the same ultrastructure and origin, the lamellar complex is generally named by most recent authors as the capsule (reviewed Zelená 1994; Bell et al. 1994).

Since many components of the ECM (e.g., proteoglycans) are extremely water soluble and thus readily extracted by conventional preparation methods for electron microscopy (Szirmai 1963), it is likely that the current picture of the structural composition of the ECM in the inner core and capsule of Pacinian corpuscle is incomplete. However, improved preservation and molecular architecture of the proteoglycans has been achieved by the addition of cationic dyes to the fixative solution prior to routine processing for electron microscopy (Chen and Wight 1984).

In the present paper, we have examined the fine structural composition of the ECM, with particular reference to proteoglycans of the inner core and capsule in the interosseal Pacinian corpuscles of the rat after application of different cationic dyes and tannic acid. The results reported here are discussed in relation to the possible functions of proteoglycans in the inner core as well as the capsule of Pacinian corpuscles.

Materials and methods

Nine adult Wistar rats of both sexes, weighing 200–250 g, were deeply anaesthetized by i.p. injection of a mixture (0.2 ml/100 g) containing xylazine (4 mg/ml) and ketamine (40 mg/ml) and perfused via the ascending aorta with 2% formaldehyde, 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The interosseal membranes were removed, the Pacinian corpuscles were isolated, cut transversely into two parts under a dissecting microscope, and immersed in different fixative solutions (see below). The experiments were carried out according to protocols approved by the Ethical Committee of the Faculty of Medicine, Brno, Czech Republic.

Ruthenium red staining

The interosseal Pacinian corpuscles of two rats were immersed in 2% formaldehyde, 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, containing 0.2% ruthenium red (Merck, Darmstadt) for 2 h at 25°C. The primary fixation was followed by an overnight rinse with 0.1 M sodium cacodylate buffer (pH 7.2) containing 0.1% ruthenium red. The material was then osmicated in 1% osmium tetroxide solution containing 0.05% ruthenium red for 2 h (Luft 1971). To improve penetration of ruthenium red into the interlamellar spaces, cryostat sections (100-µm-thick) were prepared from Pacinian corpuscles of an additional animal and incubated in the same way as described above.

Alcian blue staining

The interosseal Pacinian corpuscles that had been removed from two perfused rats were immersed for further fixation in 2% formaldehyde, 1.5% glutaraldehyde and 1% alcian blue 8GX (Sigma-Aldrich, Prague) in 0.025 M sodium acetate buffer, pH 5.8 containing 0.3 M $MgCl_2$ (the critical electrolyte concentration procedure; Scott and Dorling 1965).

Acridine orange staining

The Pacinian corpuscles that had been isolated from the interosseal membranes of two rats were immersed in 2% formaldehyde, 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 0.2% acridine orange (Sigma-Aldrich, Prague) for 2 h at room temperature. The material was rinsed three times with 0.1 M phosphate buffer (pH 7.2) containing 0.2% acridine orange for 1 h (Timar et al. 1985).

Tannic acid fixation

The isolated Pacinian corpuscles (two rats) were immersed in Zamboni's fixative solution (Zamboni and de Martino 1967) containing 2% tannic acid (EM grade, Polysciences, USA) for 5 h at room temperature. Thereafter, the samples were rinsed in sodium phosphate-buffered saline (PBS, pH 7.2) containing 2% tannic acid overnight at room temperature.

Enzyme treatment

The unfixed Pacinian corpuscles of one animal were treated with chondroitinase-ABC (5 U/ml) (Miles Laboratories, Elkhart, Ind.) in 0.2 M TRIS buffer (pH 8.0) for 30 min at 37°C prior to fixation in Zamboni-tannic acid mixture. Digestion by chondroitinase-ABC effectively removes sulfated glycosaminoglycans (Yamada 1974).

Subsequent treatment

The material from the three last types of fixation (alcian blue, acradine orange, tannic acid) was postfixed in 1% osmium tetroxide solution in 0.1 M sodium cacodylate (in the case of acridine orange, phosphate) buffer (pH 7.2) for 2 h. All specimens were dehydrated in ascending concentrations of ethanol and routinely embedded in Durcupan ACM (Fluka, Switzerland). The corpuscles were cut perpendicularly to their longitudinal axis. Semithin sections were stained with toluidine blue in order to verify the position of the Pacinian corpuscle. Ultrathin serial sections were examined under a Tesla BS-500 electron microscope without any further staining (by lead citrate or uranyl acetate).

Results

Ruthenium red

Ruthenium red positive material was found only in the structures of the inner core. Fine electron dense particles were deposited on the surface of the plasma membrane of the specialized Schwann cells and their lamellar processes. The cytoplasm of the inner core lamellae contained numerous aggregates of ruthenium red-positive granules. Similar granules with a diameter of 10–20 nm were scattered in a faintly stained interlamellar ground substance and not associated with any fibrillar material. In some sites, the dispersed granules were surrounded by an electron-lucent halo. We never observed a regular interval between deposited ruthenium red-positive granules (Fig. 1).

In some areas, the faintly stained ground substance appeared to be more condensed at the surface of the lamellae. Ruthenium red had a similar affinity to the interlamellar and cleft ECM as judged from the comparable pattern of distribution of their granules. The axolemma

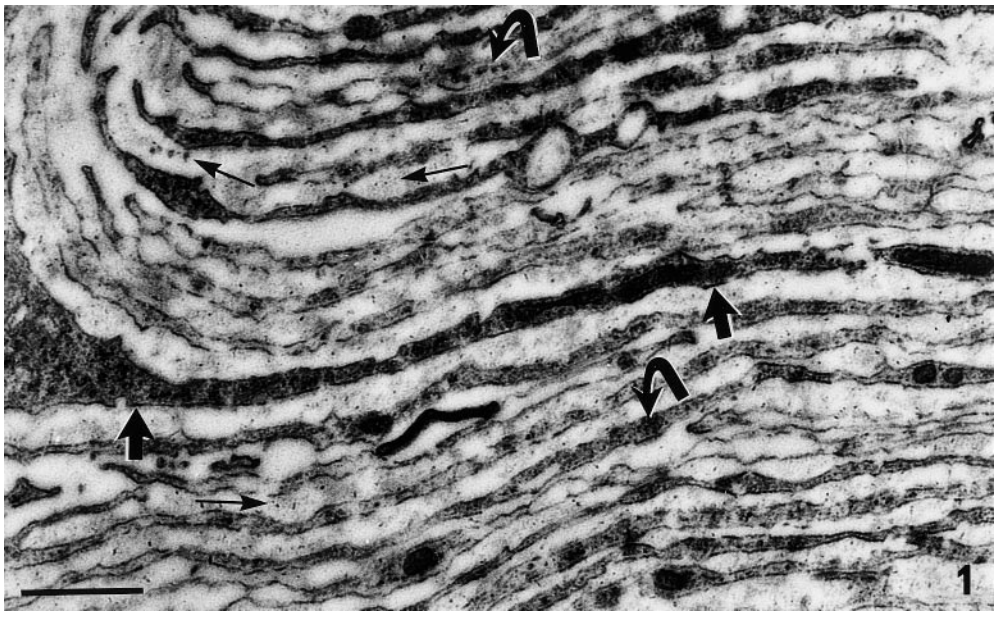

Fig. 1 Cross section through the inner core of a Pacinian corpuscle fixed in the presence of ruthenium red. Numerous granules of about 20–30 nm in diameter with a characteristic electron lucid halo are dispersed in the ground matrix (*long arrows*) and in the lamellae (*curved arrows*). A fine electron-dense material is localized in the plasma membrane, as well (*large arrows*). *Bar* 1 µm

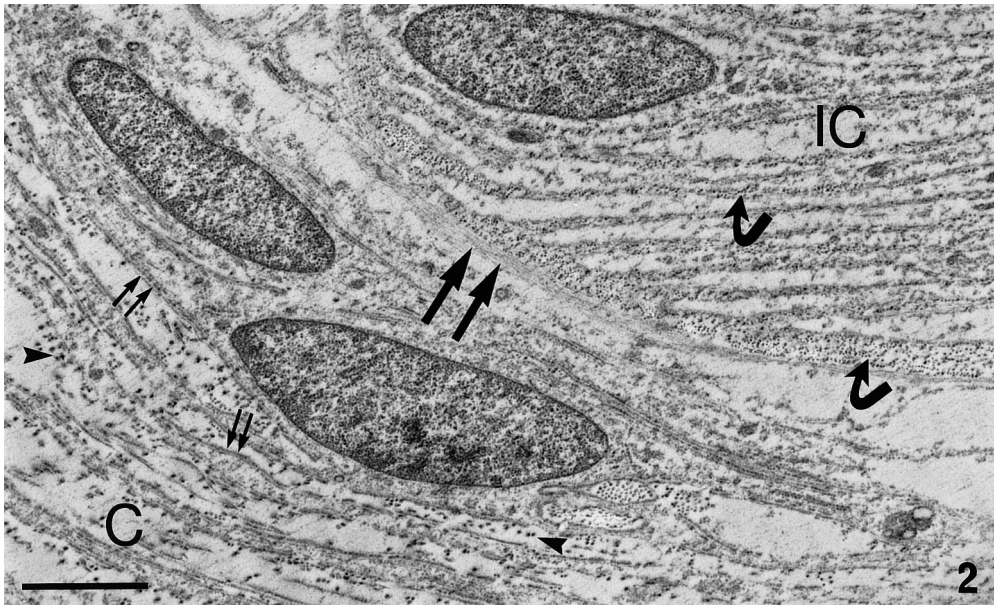

Fig. 2 Cross section through a Pacinian corpuscle at the boundary between the inner core (*IC*) and capsule (*C*) following aldehyde fixation with the addition of alcian blue and $MgCl_2$. Electron dense granules are densely distributed in narrow interlamellar spaces of the inner core (*curved arrows*) or attached to sparse collagen fibrils in the capsule (*arrowheads*). The outer surface of the inner core is covered by a layer of fine filaments (*large arrows*). The presence of fine granules provides an increased electron density to the basal lamina covering the capsular lamellae (*small arrows*). *Bar* 2 µm

of the sensory terminal did not express an enhanced electron density corresponding with ruthenium red affinity. No difference in staining pattern or intensity could be observed between Pacinian corpuscles stained “en bloc” or in their cryostat sections.

Alcian blue

The Pacinian corpuscles fixed in the presence of alcian blue displayed numerous electron dense granules. The granules were about 20 nm in diameter and densely dispersed in the inner core ECM, while the lamellae and their sparse organelles remained relatively electron lucent. In addition, the surface of the inner core was covered by a layer, 100–320-nm-thick, consisting of very fine fibrils (Fig. 2).

Fine particles with affinity for alcian blue increased the electron density of the basal lamina (about 70-nm-thick) on the external and internal surfaces of the capsular lamellae. Similar particles were also associated with the collagen fibrils that course in the interlamellar space of the capsule longitudinally and/or circularly with respect to the long axis of the corpuscle (Fig. 2).

Acridine orange

The Pacinian corpuscles fixed in the presence of acridine orange exhibited a quite different composition of the ECM

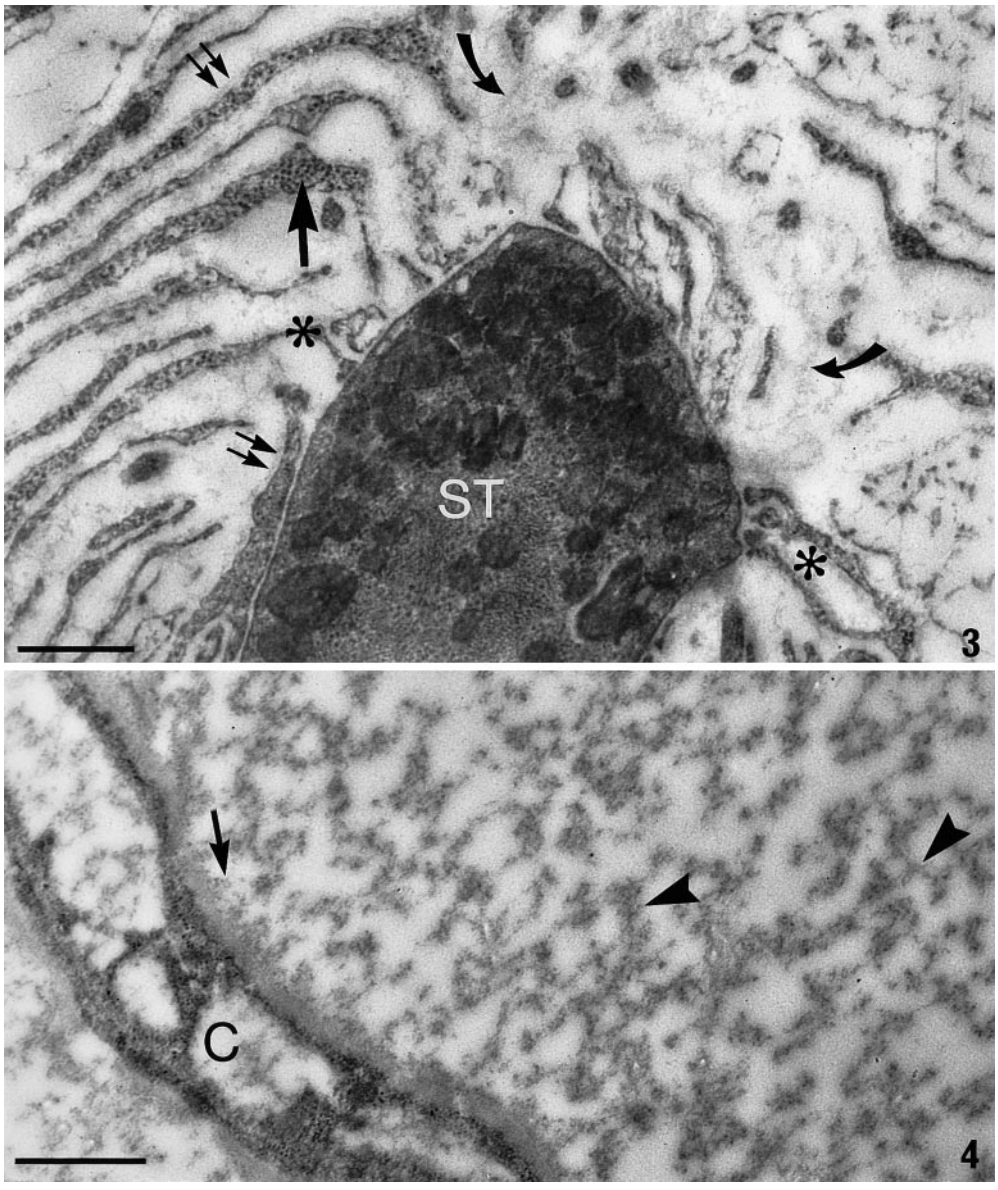

Fig. 3 Cross section through the sensory terminal (*ST*) and innermost inner core lamellae of a Pacinian corpuscle fixed in the presence of acridine orange. Some inner core lamellae are oriented perpendicularly to the surface of the sensory terminal (*asterisks*). The lamellae covered by the basal lamina (*double arrows*) are filled with numerous electron dense granules (*large arrow*). The interlamellar substance contains some fibrils (*curved arrows*). *Bar* 0.5 µm

Fig. 4 Cross section through the capsular lamella (*C*) containing electron dense particles and close interlamellar substance. A flocculent material fused with the basal lamina (*arrow*) on the surface of the lamella. Some "bottlebrush" structures can be identified (*arrowheads*). The material was fixed in the presence of acridine orange. *Bar* 0.4 µm

than those prepared with the dyes mentioned above. The cleft space of the inner core was filled with numerous filaments of various length and associated with each other.

Serial sections through the Pacinian corpuscles revealed the presence of an inner core compartment containing lamellae attached perpendicularly to the surface of the axolemma (Fig. 3; cf. Dubový and Svíženská 1989, 1993). An extended interlamellar space of this compartment contained a homogeneous material of moderate electron density that merged with the basal lamina covering the innermost lamellae (Fig. 3). The basal lamina of inner core lamellae exhibited uniform density comparable to that covering the surface of the inner core and capsule lamellae.

The interlamellar space of the capsule was filled with a flocculent material of medium electron density. The substance was composed of aggregates of very fine particles that merged irregularly with the basal lamina on the surface of lamellae. The basal lamina covering the capsule lamellae was about 70-nm-thick, and displayed the same electron density as the flocculent interlamellar material. In some places of the flocculent material, "bottlebrush" aggregates could be observed (Fig. 4).

The collagen fibrils of the cleft space and capsule were connected with a fuzzy material containing distinct filamentous material. In addition to the staining of the ECM, the application of acridine orange to the fixative solution also revealed fine electron dense particles in the cytoplasm of the inner core as well as in capsule cells and their processes (Figs. 3, 4). The axolemma of the sensory terminals displayed an increased electron density as well (Fig. 3).

Tannic acid

The addition of tannic acid to the fixative solutions resulted in such strong staining of the inner core ECM as to

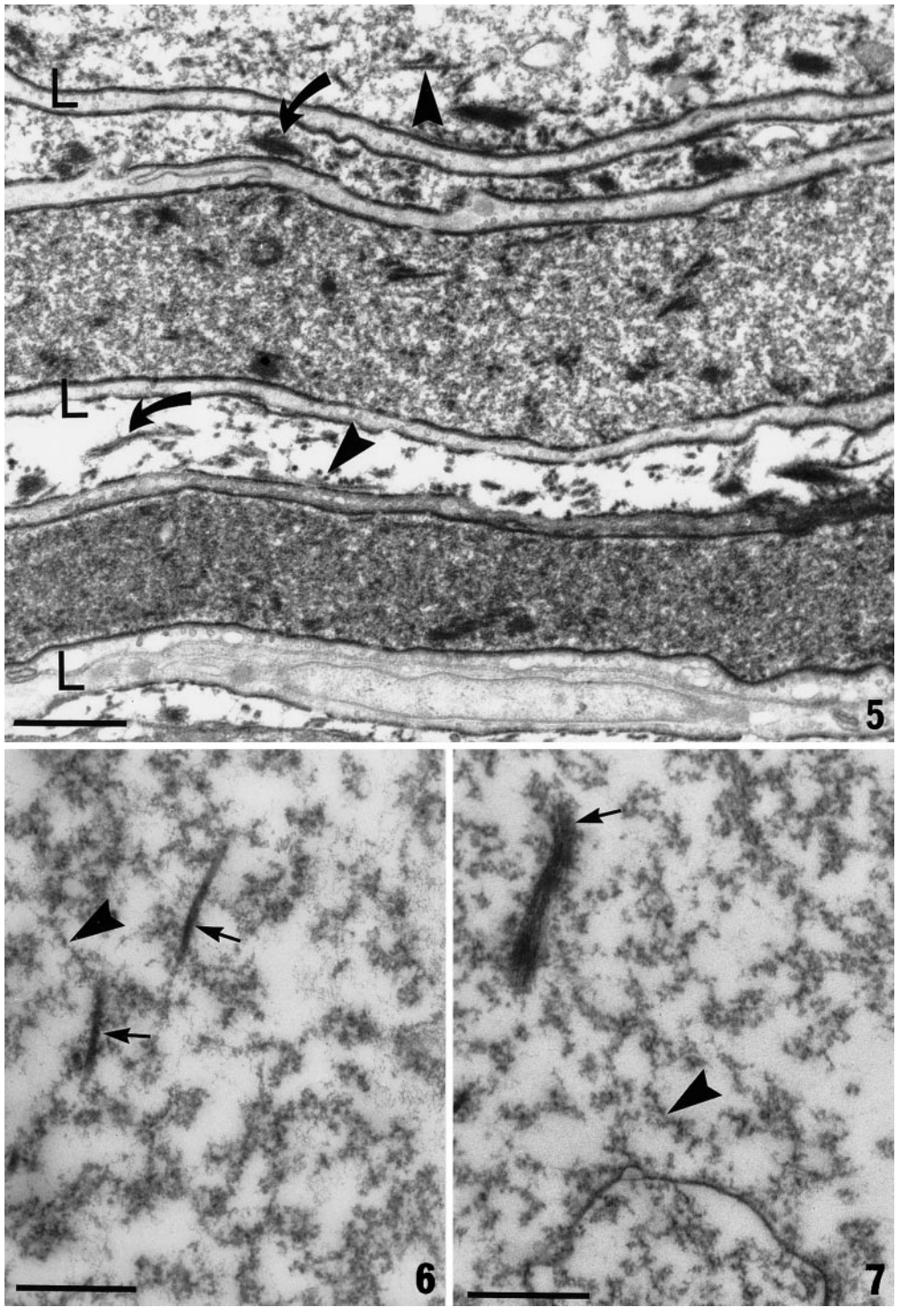

Fig. 5 Cross section through a typical part of the capsule of a Pacinian corpuscle mordant with tannic acid. The interlamellar spaces are filled with a flocculent substance of various density containing scanty collagen fibrils (*arrowheads*) and elastic fibers (*curved arrows*). The lamellae (*L*) covered by the distinct basal lamina appear to be unstained. *Bar* 1.5 µm

Figs. 6, 7 Details of the interlamellar material in the capsule of a Pacinian corpuscle mordant with tannic acid. The well-defined "bottlebrush" structures with a central cord are seen in a flocculent material (*arrowheads*). A fuzzy substance is present on the surface of the collagen fibrils (*arrows* in **6**) as well as the elastic fibers (*arrow* in **7**). *Bars* 0.5 µm

to make structural resolution impossible. In contrast to the inner core, the Zamboni-tannic acid fixative allowed excellent visualization of the basal lamina on both surfaces of the capsular lamellae and the ECM in between. The enlarged interlamellar spaces were filled with thick flocculent material of a higher electron density. Some interlamellar spaces were filled with a sparse flocculent material and contained mainly distinct collagen fibrils and elastic fibers (Fig. 5). The outermost capsular lamellae were separated by very narrow interspaces containing only collagen fibrils. No other differences between the lamellae with enlarged interspaces and those located in the superficial part of the capsule were observed.

The flocculent material was composed of individual aggregates of various shapes. These aggregates were associated with the periphery of collagen fibrils and elastic fibers. It was frequently possible to distinguish many "bottlebrush" structures with a strand in the center (Figs. 6, 7).

The basal lamina of capsular lamellae had a thickness from 66 to 73 nm and its subdivision into the lami-

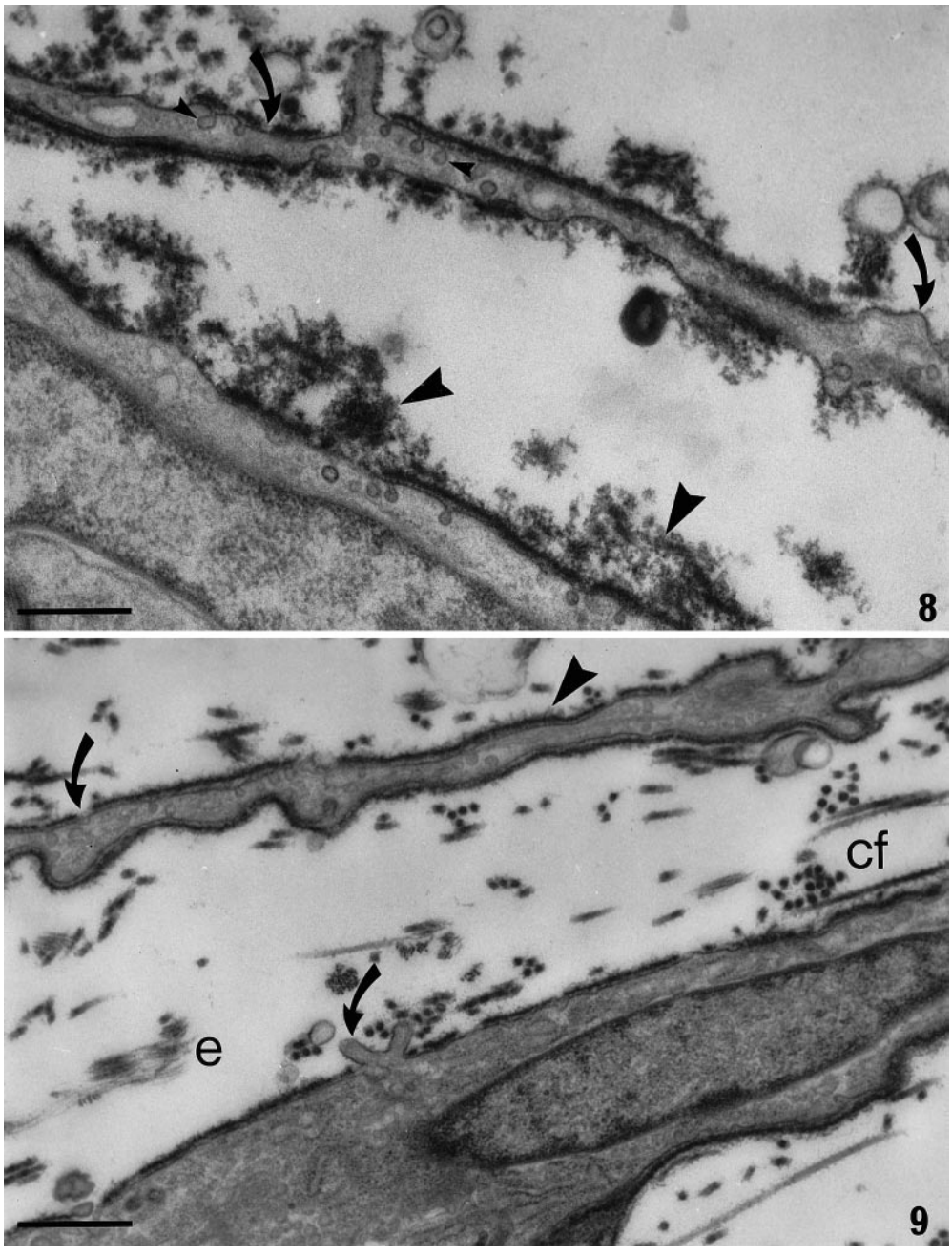

Fig. 8 Higher magnification of a cross section through the capsule of a Pacinian corpuscle mordanted with tannic acid. A flocculent dense material fused with a discontinuous basal lamina (*large arrowheads*) on both surfaces of the lamellae. The basal lamina displayed a distinct electron-dense layer corresponding to its lamina densa. Patches of the lamellar surface free of a compact basal lamina (*curved arrows*) occur in places of some protrusions but never in association with caveolae (*small arrowheads*). *Bar* 0.7 μm

Fig. 9 Cross section through the capsule of a Pacinian corpuscle treated with chondroitinase and subsequently fixed in the presence of tannic acid. The flocculent substance seen in Fig. 8 has disappeared from the interlamellar spaces but the basal lamina, collagen fibrils (*cf*) and elastic fibers (*e*) are unaffected. A fuzzy material persists on the surface of the basal lamina (*arrowhead*). Some areas of the lamellar surface lack a continuous basal lamina (*curved arrows*). *Bar* 0.7 μm

na lucida and lamina densa could be observed at higher magnification (Figs. 8, 9). The basal lamina covering was not continuous along the entire capsule lamella surface. Basal lamina-free patches were irregularly distributed on the lamellar surface. Some finger-like processes or protrusions, which emanated from the surface of the lamellae into the interlamellar space, appeared to be free of basal lamina, as well. In contrast, a consistent basal lamina covered sites where the caveolae fused with the plasma membrane and opened on the surface. However, the contents of the caveolae were never filled with electron dense substance (Fig. 8). Moreover, no other organelles of the capsular cells displayed enhanced electron density.

Digestion of the Pacinian corpuscles with chondroitinase-ABC eliminated most of the flocculent material in the interlamellar capsular spaces. The staining of collagen fibrils, elastic fibers and lamina densa of the basal laminae were, however, unchanged, and their surfaces exhibited a fine web or fuzzy material (Fig. 9).

Discussion

Cationic dye application

Proteoglycans are important components of the ECM in the nervous system (Reichardt and Tomaselli 1991).

However, they are subject to considerable dissolution and extraction during conventional preparation of specimens for electron microscopy. It has been demonstrated previously that the simultaneous use of cationic dyes such as ruthenium red, acridine orange or alcian blue with aldehyde fixation reduces the proteoglycan extraction and permits their visualization at the electron microscopic level (Chen and Wight 1984; Timar et al. 1985). Therefore, preservation of the ECM containing proteoglycans is also improved in the rat Pacinian corpuscles by using cationic dyes rather than routine aldehyde fixation.

Proteoglycans appear to have a large structural diversity reflecting their wide biological functions (Hardingham and Fosang 1992). The present results demonstrate proteoglycans in both the inner core and the capsule of Pacinian corpuscles, but in a somewhat different pattern and appearance depending on which cationic dye was used. Nevertheless, a different composition of proteoglycans in the ECM of the inner core and capsule could not be excluded. Previous ultrastructural studies of the arterial wall had shown that fixation in the presence of the higher-molecular weight dyes (e.g., alcian blue or ruthenium red) results in a predominantly granular appearance of the proteoglycans, while the lower-molecular weight dye (e.g., acridine orange) maintains proteoglycans as filamentous structures (Chen and Wight 1984). Our findings in Pacinian corpuscles are well in line with these observations. In addition, the tannic acid mordant also preserved the filamentous appearance of proteoglycans very well (Figs. 6, 7).

Ruthenium red and alcian blue had a predominant affinity to proteoglycans in the inner core of Pacinian corpuscles but displayed no (ruthenium red) or minimal (alcian blue) staining of the capsular ECM. The affinity of ruthenium red and alcian blue to the inner core ECM is consistent with previous observations that the pattern of alcian blue staining in Pacinian corpuscles is unchanged at different electrolyte concentrations and following hyaluronidase treatment (Halbhuber 1965; Dubový and Svíženská 1993). Therefore, it was suggested that the inner core of Pacinian corpuscles contains dermatan and heparan sulfates (Dubový and Svíženská 1993).

The use of whole corpuscles or cryostat sections had no influence on the amount of ruthenium red-positive granules in the inner core. They were deposited in the plasma membrane and cytoplasm of the inner core lamellae. Comparable intralamellar staining was observed with acridine orange while alcian blue provided exclusively extracellular staining. Some differences in the affinity of the cationic dyes used to the plasma membrane including the axolemma and to the cytoplasm probably reflect a polymorphic form of proteoglycans (Jalkanen et al. 1991; Wight et al. 1991).

It has been shown previously that acridine orange can bind specifically to glycosaminoglycan compounds and has a higher affinity for glycosaminoglycans than for nucleic acids at low concentration. This dye is also suitable for the demonstration of cell surface glycosaminoglycan at the ultrastructural level (Timar et al. 1985). Application of acridine orange revealed a filamentous material in the inner core cleft but most of the ECM material had a flocculent and homogeneous appearance. Our results suggest that the inner core lamellae, or at least those having a perpendicular attachment to the sensory terminal, are covered by a basal lamina-like material with glycosaminoglycan features. In addition, an enhanced electron density of the axolemma of sensory terminals after acridine orange-aldehyde fixation indicates the presence of membrane-bound glycosaminoglycans in this part of the sensory axon.

Tannic acid mordant

A method employing tannic acid additives in aldehyde fixation has been used to stabilize cellular structures (Wagner 1976; Kalina and Pease 1977) including microfilaments (LaFountain et al. 1977). The tannic acid can be considered as a stain mordant that fixes the ECM components more effectively than aldehyde fixation and subsequent heavy metal treatment (Simionescu and Simionescu 1976).

When tannic acid was used with aldehyde fixation in the present study, an enhanced electron density of the capsular ECM of Pacinian corpuscles was observed, while the appearance of intracellular structures including caveolae remained unaffected. The finding of typical “bottlebrush” structures suggested that tannic acid preserved the structural identity of capsular glycosaminoglycans like acridine orange. Digestion with chondroitinase prior to fixation effectively removed the material with tannic acid affinity in the capsular interlamellar space. This confirms the glycosaminoglycan nature of this material and indicates that sulfated glycosaminoglycans are the predominant type in the capsule of the Pacinian corpuscle.

Inner core proteoglycans and their possible implication in maintenance of the ionic microenvironment

Both ruthenium red and alcian blue are used for routine demonstration of polyanionic sites (Kanwar and Farquahar 1979; Reale et al. 1985). However, ruthenium red- or alcian blue-positive particles were never found at the regular distances that correspond to the position of polyanionic sites in the basal lamina of Schwann cells (Yokota et al. 1983). Our electron microscopic observations suggest that the polyanionic sites are dispersed throughout the inner core ECM to ensure its function (see below).

The diameter of alcian blue granules in the inner core ECM is very similar to the diameter of collagen fibrils in this compartment of the Pacinian corpuscles (15–20 nm, see Munger et al. 1988). Moreover, the alcian blue-positive granules were also attached to the collagen fibrils of

the capsule. A connection between proteoglycans and collagen fibrils has been demonstrated previously in various tissues and is considered to be a general situation (Myers et al. 1973; Scott 1988). Therefore, the polyanionic sites provided by proteoglycans seem to be related to the collagen fibrils that are densely packed in the narrow interlamellar spaces of the inner core.

The sensory terminal is the site of mechano-electric transduction in Pacinian corpuscles (Loewenstein 1971). The transduction of mechanical stimuli to a generator potential is accompanied by large ionic fluxes. Based on structural and functional similarities between the node of Ranvier and the inner core of the corpuscles, it was suggested that the inner core lamellae and their ECM are involved in the maintenance of the ionic microenvironment around the sensory terminals (Dubový 1986; Dubový and Malinovský 1986; Dubový and Svíženská 1993). The large amount of proteoglycans in the inner core ECM probably reflects the significance of these molecules in the regulation of ionic homeostasis around the sensory terminals of Pacinian corpuscles. The use of alcian blue revealed a fine fibrous material forming a fibrillar meshwork (web) on the surface of the inner core where the basal lamina has been described following routine preparation of specimens for electron microscopy (Idé and Hayashi 1987; Munger et al. 1988). This meshwork of filaments may correspond to proteoglycans, especially heparan and chondroitin sulfate proteoglycans that are common components of the basal lamina (Leblond and Inoue 1989). The layer on the surface of the inner core is, however, thicker than the basal lamina of Schwann cells of nerve trunks or the basal lamina found on the surface of the inner core lamellae in acridine orange treated material. It seems that this layer may contribute to isolation of the microenvironment of the inner core space together with the intermediate lamellae that are joined by the tight junctions (Idé and Hayashi 1987). This separation of fluids and ions between the inner core and capsule has been considered to be important for normal function of Pacinian corpuscles (reviewed Bell et al. 1994).

The capsular proteoglycans

The Pacinian corpuscle is an encapsulated quickly-adapting sensory nerve ending that provides the basis for the sensation of high-frequency vibrations and moving touch. The capsule acts as a filter to restrain low frequency vibrations (less than 100 Hz) and to be maximally responsive at 250–300 Hz (Loewenstein 1971).

The capsular ECM contains collagen fibrils and elastic fibers to sustain its flexibility. Since proteoglycans are extremely hydrophilic due to their anionic charge, they are capable of providing the required turgor pressure and viscoelasticity of the ECM (Chen and Wight 1984). The proteoglycans in the interlamellar spaces probably contribute to the preservation of capsule flexibility by operating as a mechanical filter.

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