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Full-length paper

The position and morphology of honeycombs in normal skeletal muscle fibres of the healthy frog

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Abstract Honeycombs are regularly arranged networks of tubules in continuity with the T-system of the skeletal muscle fibre. Their occurrence is usually described in pathologically modified or cultivated muscle fibres. Here we describe the occurrence of honeycombs in macroscopic normal muscle fibres of healthy frogs. We characterize their light- and electron-microscopic features and represent their relationships to motor end plates, fibre nuclei and myofibrils. In these normal muscle fibres of healthy frogs, the honeycombs are connected to subsynaptic folds and the T-system. Their regional occurrence is discussed with respect to regional differences in the regulation of the membrane metabolism. Since we can demonstrate them in healthy animals, we do not see any basis why their occurrence should be related to pathological modifications of the muscle fibre.

Keywords lanthanum, honeycomb, T-system, motor end plate, light microscopy, electron microscopy

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Introduction

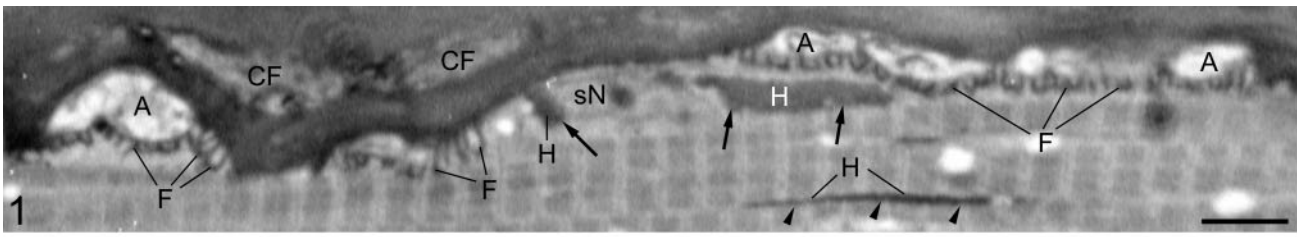
Recent ultrastructural investigations of skeletal muscle fibres showed regularly arranged networks of membranous systems in continuity with the T-system, which were designated as honeycombs (for references see [1]). They are found in denervated and pathologically modified muscle fibres [2] and in degenerated anuran tadpole tail muscle fibres during natural morphogenesis [1]; however, they can be observed also in cultivated muscle fibres [3–8]. Structurally modified mitochondria and accumulations of lysosomes are described simultaneously with the occurrence of these honeycombs [9–14]. Currently, honeycombs are taken as an expression of muscle degeneration and as an indicator for an abnormal T-tubule proliferation.

During our ultrastructural search for connections between subsynaptic folds of the motor end plate and the T-system of the healthy frog [15], we observed honeycombs in ultrastructural normal muscle fibres. Due to the dense staining of honeycombs through the lanthanum incubation, we can show them for the first time with the light microscope and compare their features with corresponding electron microscopic images of ultrathin (80 nm) and semi-thin sections (0.15–1 μm). Their connections to subsynaptic folds were investigated and compared with connections of T-tubules to subsynaptic folds of the frog motor end plate.

Methods

Muscle fibres and their motor end plates of the sartorius muscle of adult frogs (*Xenopus laevis* ($n = 4$) and *Rana temporaria* ($n = 3$)) were examined. The frogs were anaesthetized with MS222 (Sandoz Co., Basel, Switzerland) and decapitated. The legs were exarticulated at the pelvic joint. In a Petri dish filled with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, the skin of the leg was removed, the superficial sartorius muscle exposed and its fascia removed completely. Then, the entire leg was immersion-fixed in the same fixative for 1 h. Subsequently, the muscle was divided into small strips of tissue and allowed to remain in the fixative agent for a further 2 h. After washing in cacodylate buffer, the muscle samples were postfixed in 1% osmium tetroxide in 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer for 3 h.

Since lanthanum does not infiltrate the cell [16], it is an appropriate marker for the representation of the extracellular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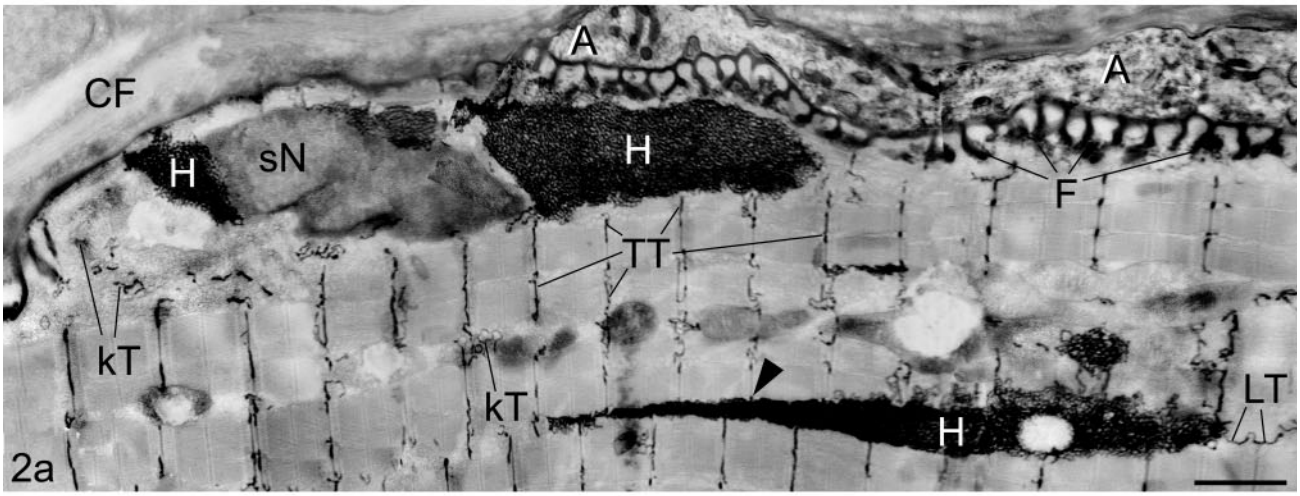
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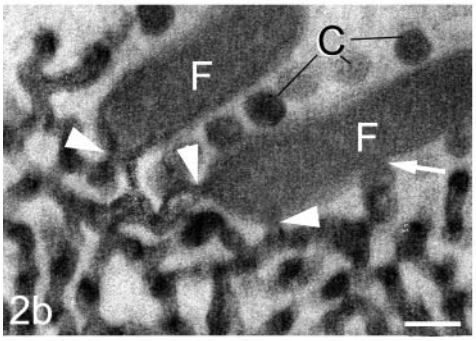
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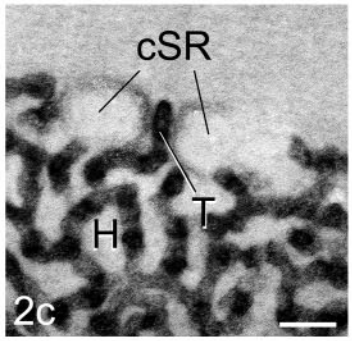
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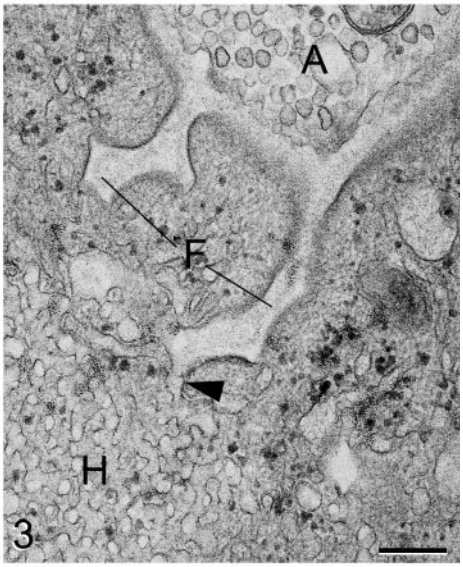
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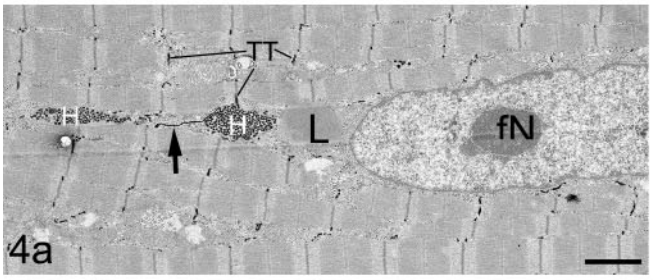
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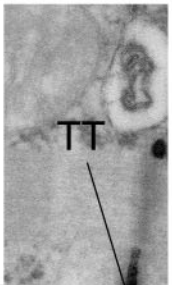
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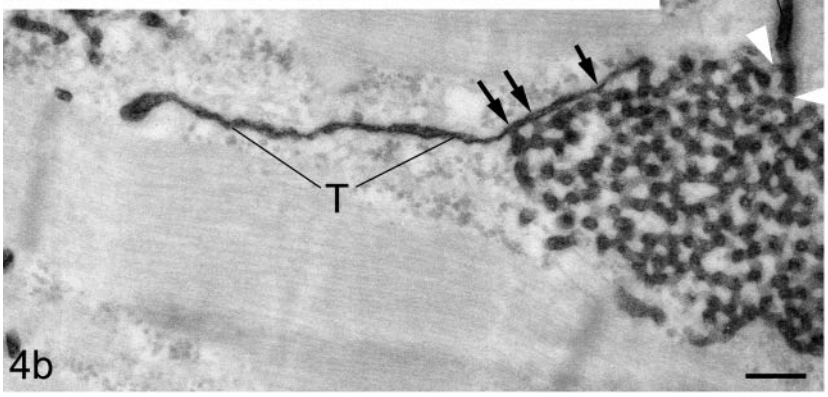
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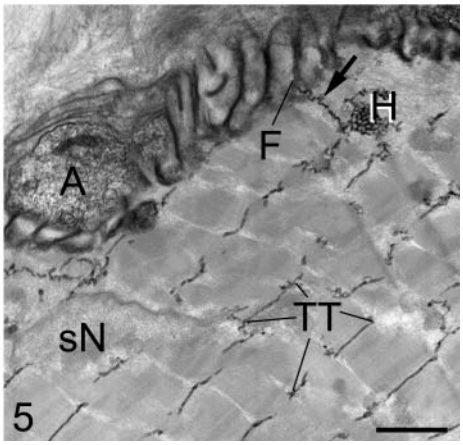
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space, i.e. the T-system, the subsynaptic folds and the caveolae of muscle fibres. For lanthanum incubation, the muscle fibres were incubated for 1.5 h in 1% lanthanum nitrate in 0.1 M cacodylate buffer on an agitator at room temperature. Following dehydration by means of an ascending series of alcohol solutions, the tissue was embedded in EPON. For light-microscopic investigation, semi-thin sections were stained according to Richardson *et al*. [17]. Ultrathin sections of muscle samples without lanthanum incubation were stained with uranyl acetate and lead citrate [18]. Ultrathin sections (80 nm) and semi-thin sections (0.15–1 μm) of lanthanum-incubated muscle samples were examined in the unstained state. All sections were examined by the electron microscope Leo912 Omega (Zeiss, Oberkochen, Germany).

Results

Honeycombs could be observed in the area of motor end plates of the examined frog species (Figs 1–5). Through the lanthanum incubation, the subsynaptic folds and the honeycombs, but not the T-system, could be observed with the light microscope (Fig. 1). First, with the aid of the electron microscope, the T-system (Figs 2a, 2c, 4a, 4b and 5) and the caveolae (Fig. 2b) appeared just as intense as the honeycombs (Figs 2–5) and the subsynaptic folds (Figs 2a and 2b). The lanthanum incubation leads to a negative contrast of the collagen fibre bundles in the extracellular space (Figs 1 and 2a). The marker fills the subsynaptic folds and migrates into honeycombs, which lie in proximity to these subsynaptic folds (Figs 2a and 5). The lumen transition between the subsynaptic fold and the honeycomb, in the shape of tubules or beaded caveolae, becomes visible as a result (Fig. 2b). This transition was hard to recognize without lanthanum incubation (Fig. 3). Honeycombs, which pass over into the T-system (Figs 2c, 4a and 4b), were also observed in sarcoplasm accumulations around muscle fibre nuclei (Fig. 4a). These honeycombs are connected most directly with the T-system, so that they are shared directly at the triad formation. Emergent tubules of the honeycombs, however, at first can run longitudinally and, thus, form a part of the longitudinal T-system (Figs 4a and 4b). The net-like interconnected tubules (Figs 2b, 2c and 4b) of the honeycombs were determined to have a diameter of 30 nm, which enlarges up to 50 nm at the ramifications. Differences in the dimensions between honeycombs in the area of subsynaptic folds and/or the subsarcolemmal outside of motor end plates and in the area of muscle fibre nuclei could not be found.

Discussion

The investigation has shown that honeycombs can be represented also in normal skeletal muscle fibres of healthy frogs. As shown with the electron microscope, they can be filled with lanthanum and observed as intensely as the subsarcolemmal caveolae and the T-system. The regular tubular net-like honeycomb structure is so closely woven, in contrast to the tubular network of the T-system in the motor end plate sarcoplasm of frogs [15] and mammals [19], that honeycombs can be seen with the light microscope after lanthanum incubation. The similarity of the lanthanum precipitates in these areas, i.e. T-system, caveolae and honeycombs, means that the same conditions for their formation must dominate there. The T-system contains a matrix of proteoglycans [20], which could act as binding sites for lanthanum [21,22]. On the other hand, it can be shown that the formation of lanthanum precipitates on the cell

Fig. 1 Detail of a motor end plate in the sole plate area. Lanthanum incubation allows the honeycombs (H) to be observed with the light microscope. On the one hand they are observed directly below the subsynaptic folds of the motor end plate (arrow) and on the other they are between the myofibrils of the muscle fibre (arrowhead). Abbreviations: sN, sole plate nucleus; A, axon terminal; F, subsynaptic folds; CF, collagen fibre bundle. *Xenopus laevis*; lanthanum incubation; semi-thin section (500 nm); light microscope. Bar = 5 μm.

Fig. 2 (a) Same detail as in Fig. 1 in one of the consecutive semi-thin sections viewed with the electron microscope. The transverse T-system (TT) as well as the subsynaptic folds (F) and the honeycombs (H) are filled with lanthanum. In the sarcoplasm area of the sole plate nucleus (sN), as well as between myofibrils, a tangled T-system can be recognized (kT). The arrowhead indicates transition of a honeycomb into the T-system (see detail in (c)). Abbreviations: LT, longitudinal T-system; CF, collagen fibre bundle. *Xenopus laevis*; lanthanum incubation; unstained; section thickness = 300 nm. Bar = 1 μm. (b) The section following on from that in (a), showing the tubular (arrowhead) and beaded caveolar (arrow) transition between the lanthanum-filled subsynaptic folds (F) and the cross-linked lanthanum-filled tubular system of the honeycomb. Abbreviation: C, caveolae. *Xenopus laevis*; lanthanum incubation; unstained; section thickness = 150 nm. Bar = 100 nm. (c) Detail from (a) in the region of the arrowhead. A tubule (T) of the lanthanum-filled honeycomb (H) is directly connected to a triad. Abbreviation: cSR, cisternal sarcoplasmic reticulum. *Xenopus laevis*; lanthanum incubation; unstained; section thickness = 150 nm. Bar = 100 nm.

Fig. 3 Section of a motor end plate. One subsynaptic fold (F) opens (arrowhead) to the tubular system of a honeycomb. Abbreviations: A, axon terminal; H, honeycomb. *Rana temporaria*; without lanthanum incubation; section stained; section thickness = 80 nm. Bar = 200 nm.

Fig. 4 (a) Longitudinal section through a skeletal muscle fibre. Honeycombs (H) are in the sarcoplasm area of the fibre nucleus (fN). Abbreviations: L, lipid droplet; TT, T-system. *Rana temporaria*; lanthanum incubation; section stained; section thickness = 150 nm. Bar = 2 μm. (b) Detail of the honeycomb of (a). A longitudinally aligned tube (T) originating (arrow) in a honeycomb runs in the direction of the next Z stripe. The arrowhead indicates the connection of the honeycomb with the T-tubule (TT) of a triad. *Rana temporaria*; lanthanum incubation; section stained; section thickness = 150 nm. Bar = 250 nm.

Fig. 5 Longitudinal section of a skeletal muscle fibre in the end plate area. A meandering tube (arrow), originating in a subsynaptic fold (F), runs in the direction of the honeycomb (H) with which it is in contact. Abbreviations: A, axon terminal; TT, T-system; sN, sole plate nucleus. *Rana temporaria*; lanthanum incubation; section stained; section thickness = 300 nm. Bar = 1 μm.

surface cannot be prevented by proteolytic or mucolytic enzymes, but through phospholipases [23]. This relates to binding of the lanthanum to phosphorylated lipid components of the membrane [23].

The openings of the honeycombs to subsynaptic folds of the motor end plate are formed similarly to the openings of the T-system to the subsynaptic folds, so that honeycombs must be considered as part of this membrane system [15]. Due to these connections, they should be involved in the voltage changes of the T-system. According to their extension they could regionally contribute to the membrane surface of the muscle fibre, which leads to a reduction of the passive and capacitive membrane resistance. Besides these passive resistances, the voltage changes within the T-system [24] and in analogy to that in the honeycombs also must be considered. Whether the regional accumulation of T-system membranes in the shape of honeycombs has an electrophysiological effect can first be shown by new physiological experiments.

After depolarization of the T-system membrane, its endogenous phosphatidylinositol-4,5-biphosphate (PIP2) is catalysed to inositol-1,4,5-trisphosphate (IP3) [25], which intervenes in the gene regulation of myotubes by the triggering of Ca^{2+} waves [26]. The formation of honeycombs, i.e. their regional dimension and their possibility of PIP2 liberation, can also be the cause for regional differences in the effectiveness of the PIP2–IP3 regulation cascades.

In myoblastic cell lines the formation of honeycombs was associated with developing interactions between amphiphysin and PIP2 [8]. It would be possible, therefore, that the formation of honeycombs goes along with the binding of PIP2 to lipids of the T-system membrane, which by interaction with amphiphysin is shaped into honeycombs. The observation that honeycombs are found only at individual nuclei of normal muscle fibres means that there are regional differences in the regulation of the production of T-tubules, amphiphysin and PIP2, as was described also for other muscle gene products [27,28]. Our findings in healthy animals and/or in healthy muscle fibres with well-developed motor end plates do not support a direct relation between the occurrence of honeycombs and pathological processes of frog muscle fibres. They can be considered, however, as regionally limited adaptation processes of the muscle fibre.

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