DIHYDROPYRIDINE RECEPTORS IN SKELETAL MUSCLE WITH COMPARATIVE REFERENCE TO MUSCLE DEVELOPMENT AND EXERCISE IN MOUSE AND SALMON

Abstract in Finnish
SATU MÄNTÄRΙ

DIHYDROPYRIDINE RECEPTORS IN SKELETAL MUSCLE WITH COMPARATIVE REFERENCE TO MUSCLE DEVELOPMENT AND EXERCISE IN MOUSE AND SALMON

Academic Dissertation to be presented with the assent of the Faculty of Science, University of Oulu, for public discussion in Kuusamonsali (Auditorium YB210), Linnanmaa, on May 27th, 2005, at 12 noon

OUЛУN YLIOPİSTO, OULU 2005
Abstract

The dihydropyridine receptor (DHPR) in the skeletal muscle plasma membrane functions as a voltage sensor for excitation-contraction coupling. In the present work the expression and special features of DHPR were studied under various conditions. In order to localize and visualize the DHPRs, a method using fluorophore-conjugated dihydropyridine molecules as a probe was developed. In addition, different laboratory assays and electrophysiological measurements were used to study the expression of the myofibrillar proteins, force production of the muscle and conduction velocity of the plasma membrane.

During the postnatal development of mouse skeletal muscle the density of DHPR increased. By the time of DHPR appearance, the expression of sodium channels had started and the typical assembly of basic structural components and membrane compartments was clearly detectable.

According to the histochemical analysis, the DHPR was selectively expressed in type IIA muscle fibres of mouse. In addition to the fibre type specificity, the uneven distribution of DHPRs was also seen at the muscle level. The attenuation of the contraction force after addition of DHPR blocker was largest in muscles with a high percentage of type IIA fibres. In fish muscles, the distribution of DHPR was homogenous and the density between different fibre types was quite similar.

In gastrocnemius muscle of mouse, the density and mRNA expression of DHPR increased significantly by 21.5 and 66.8%, respectively, after a 15-week aerobic exercise programme. The increase correlated significantly with the raise in % myosin heavy chain IIa isoform. In thigh and heart muscles, no significant changes were observed.

In fish, the environmental change following hatchery release and downstream migration of 46.5 km induced an increase in the DHPR density in swimming muscles. Furthermore, a transition of phenotypic profile from fast-to-slow fibres was observed.

Taken together, the present data provide evidence for the fact that the expression of DHPR increases during postnatal development. Moreover, the expression correlates with a specific fibre-type metabolism, thus having an impact on the overall contractile properties of the muscle. This is further manifested as an increase in the DHPR density after endurance training in mammalian as well as in fish muscle. In addition, a strong correlation exists between the level of muscle activity and the density of DHPR.

Keywords: DHPR, E-C coupling, fibre types, fish swimming musculature, L-type calcium channel
Mänttäri, Satu, Vertaileva tutkimus kehittymisen ja rasituksen vaikutuksista dihydropyridiinireseptorien
Luonnontieteellinen tiedekunta, Biologian laitos, Oulun yliopisto, PL 3000, 90014 Oulun yliopisto
2005
Oulu, Finland

**Tiivistelmä**

Poikkipiuonainen lihaksen solukalvolla esiintyvä dihydropyridiini (DHP) reseptori toimii jännitesensorina lihaksen ärsytys-supistus kytkennässä. Tässä työssä tutkittiin DHP reseptorin erityispiirteitä sekä erilaisen fysiologisten tekijöiden vaikutusta reseptorin ilmenemiseen. DHP reseptorien visualisointiin kehitettiin histologinen värjäysmenetelmä, jossa merkkiaineena toimi fluorofori-konjugoitu DHP molekyyli. Lisäksi työssä tutkittiin lihaksen proteiinien ekspressiota, lihaksen voimantuottoa sekä solukalvon johtonopeutta erilaisen määritysmenetelmien ja elektrofysiologisten mittausmenetelmien avulla.


15 viikon aerobinen harjoittelu lisäsi sekä DHP reseptorin proteiini- että mRNA-ekspresiota tilastollisesti merkitsevästi (21,5 ja 66,8 %) hiiren kantalihaksessa. Ekspresion kasvu korreloi merkitsevästi samanaikaisesti tapahtuneen myosini isomuoto IIa määrän kasvuun kanssa. Reisi- ja sydänlihaksessa merkittäviä muutoksia ei havaittu.

Kalan uintilihaksissa DHP reseptorien tiheys kasvoi vapauttamisen jälkeisen ympäristön muutoksen ja 46,5 km pituisen vaelluksen jälkeen. Lisäksi lihasten solutyyppikoostumuksessa tapahtui muutos kohti hitaasti supistuvia solutyyppiä.

Yhteenvetona voidaan todeta, että saatujen tulosten perusteella DHP reseptorien ekspresio kasvaa syntymän jälkeen hiiren poikkipuuonaisessa lihaksessa. Solujen erilaistuessa ekspresion korreloi solutyyppikoostumuksen kanssa vaikuttaen edelleen lihaksen supistumisomaisuuksiin. Tästä johtuen myös kestävyysharjoittelun seurauksena DHP reseptorien määrä kasvaa sekä nisäkkään että kalan lihaksissa. Erityisesti lihaksen aktiivisuudella on merkitystä DHP reseptorin ekspresioon.

**Asiasanat:** DHPR, L-tyyppin kalsiumkanava, lihaksen supistuminen, lihassolutyypit, uintilihakset
To Tommi, Joni and Sonja
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I want to express my special thanks to the following experts in the laboratory: Riitta Harjula, whose knowledge and skill in microscopy techniques I admire, and from whom I learned so much; Marja-Liisa Martimo, who taught me many analytical methods and helped several times with sampling and analysis; Minna Orreveteläinen, who helped with
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With my heart full of love, I thank you for keeping my feet on the ground and for your sincere love. You are the most wonderful and valuable things in my life.

April 2005, Oulu

Satu Mänttäri
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCIP</td>
<td>Bromo-4-Chloro-3-Indolyl Phosphate Mono-(-Toluidinium) Salt</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>Cacna1S</td>
<td>L-type voltage-dependent calcium channel, alpha 1 skeletal muscle type subunit</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium induced calcium release</td>
</tr>
<tr>
<td>CLQ</td>
<td>Calsequestrin</td>
</tr>
<tr>
<td>DHP</td>
<td>Dihydropyridine</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>E-C</td>
<td>Excitation-contraction</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FG</td>
<td>Fast glycolytic fibre type</td>
</tr>
<tr>
<td>FOG</td>
<td>Fast oxidative glycolytic fibre type</td>
</tr>
<tr>
<td>GAS</td>
<td>Gastrocnemius muscle</td>
</tr>
<tr>
<td>GLU</td>
<td>Gluteus maximus muscle</td>
</tr>
<tr>
<td>InsP₃</td>
<td>Inositol (1,4,5)-triphosphatase</td>
</tr>
<tr>
<td>LTS</td>
<td>Longitudinal tubular system</td>
</tr>
<tr>
<td>MDF</td>
<td>Myogenic determination factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MHCe</td>
<td>Embryonal isoform of myosin heavy chain</td>
</tr>
<tr>
<td>MHCpn</td>
<td>Perinatal isoform of myosin heavy chain</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro blue tetrazolium</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>RF</td>
<td>Rectus femoris muscle</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>S1</td>
<td>Subfragment 1</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinic dehydrogenase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic (endoplasmic) reticulum Ca-ATPase</td>
</tr>
<tr>
<td>SO</td>
<td>Slow oxidative fibre type</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>T-tubule</td>
<td>Transverse tubule</td>
</tr>
<tr>
<td>Tn</td>
<td>Troponin</td>
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</table>
List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals, and some original unpublished data.


Contents

Abstracts
Acknowledgements
Abbreviations
List of original papers
Contents
Preface
1 Review of the literature ................................................................. 19
  1.1 Skeletal muscle cell ................................................................. 19
    1.1.1 Myogenesis ........................................................................ 19
  1.1.2 Morphogenesis .................................................................... 20
  1.1.3 Origin and diversity of muscle fibre types.......................... 22
  1.1.4 Structural basis of muscle contraction ............................... 23
    1.1.4.1 Myofibril ........................................................................ 23
    1.1.4.2 The sarcoplasmic reticulum and the transverse tubules .... 23
    1.1.4.3 Ultrastructure of the triad .............................................. 24
  1.1.5 Muscle contraction .............................................................. 26
    1.1.5.1 Excitation-contraction coupling ................................. 26
  1.2 Voltage-gated sodium channels in skeletal muscle ................. 27
  1.3 Dihydropyridine sensitive calcium channels in skeletal muscle .. 28
    1.3.1 Dihydropyridine receptor ............................................... 29
      1.3.1.1 Structure and molecular pharmacology ...................... 29
      1.3.1.2 Dual function of the dihydropyridine receptor .......... 30
      1.3.1.3 DHPR adaptation ...................................................... 31
  1.4 Aims of the study ................................................................. 33
  2 Aims of the study.................................................................. 33
  3 Material and methods ............................................................. 34
    3.1 Animals .............................................................................. 34
    3.2 Electrophysiological measurements ................................. 34
      3.2.1 Conduction velocity measurements ......................... 34
      3.2.2 Force measurements .................................................. 34
    3.3 Endurance training ......................................................... 35
    3.4 Laboratory assays ............................................................ 35
Preface

Calcium ions play an essential role as second messengers in nearly all cell types, being the primary regulator of skeletal muscle contraction. Since the presence of calcium ions is fundamental for the initiation of contraction, it is very important to understand the basic mechanisms by which Ca$^{2+}$ flows to the cytosol.

Close attention has been focused in recent years on the specific mechanisms involved in excitation-contraction coupling - a series of events linking the action potential to contraction via release of calcium ions. A major breakthrough in this field came when Schneider and Chandler (1973) discovered that voltage sensors in the transverse (T) tubule membrane may physically interact with the calcium channels in the sarcoplasmic reticulum. It was later found that the gating charge movements were those of dihydropyridine receptors embedded in the T-tubule membrane.

One of the characteristics of skeletal muscle tissue is the structural variability of its muscle fibres. It is well established that different fibre types have different mechanical and energetic properties. The functional diversity of fibre types has been further related to alterations in relative proportions of membrane structures and different densities of contractile proteins. Despite intensive study of fibre type properties, very little is known about the processes which in different fibre types lead from electrical membrane excitation to contraction.

In the present thesis, I focus on the distribution of the dihydropyridine receptor in order to better understand the excitation-contraction coupling properties in different types of muscle fibres.
1 Review of the literature

Muscle tissue represents an extreme specialization of the cellular mechanisms of movement. Adult skeletal muscle is a unique tissue composed of differentiated muscle fibres with an organized array of structural proteins. The highly organized structure, from the gross anatomy down to the molecular level, enables a rapid and efficient generation of movement and force production. Skeletal muscle tissue also demonstrates a remarkable plasticity, capacity to adapt to a variety of external stimuli. The skeletal muscle tissue is an excellent model of a biological structure which integrates functions such as motion, maintenance of posture and heat production. In this chapter skeletal muscle characteristics of mammalian muscle tissue are reviewed.

1.1 Skeletal muscle cell

1.1.1 Myogenesis

In mammals, myogenesis begins with the appearance of somitomeres and somites derived from the paraxial mesoderm of the embryo (Kaufman & Bard 1999). The mesodermal cells undergo frequent mitoses until either the stem cell, the daughter cell or both escape from the cycle to become postmitotic myoblasts. Myosatellite cells represent a unique group of stem cells capable of withdrawing from the cell cycle and entering a quiescent phase. These cells re-enter the cycle in response to muscle trauma and various disease states and act as a source of nuclei during muscle growth and regeneration (Schultz 1989).

After escaping from the cell cycle, postmitotic myoblasts fuse with each other to form multinucleated, spindle-shaped cells called primary myotubes (Moss & Strohman 1976). During maturation, secondary and tertiary (only in the muscles of larger mammals such as man) myotubes are formed when primary myotubes and adjacent myoblasts fuse. Once the fusion has occurred, the synthesis of major structural proteins and the formation of membrane structures are initiated. The muscle cells are called myofibers not until all the nuclei have a subsarcolemmal position (Dauncey & Gilmour 1996, Swatland 2004).
The factors controlling muscle cell proliferation, termed myogenic determination factors (MDFs), are molecules that are able to convert any nonmuscle cells to a myogenic phenotype (Hauschka 1994). Most of the MDFs are transcriptional factors such as cell-type specific promoters or enhancers (Firulli & Olson 1997). The four known vertebrate MDFs, i.e., MyoD, myogenin, Myf-5 and MRF4, are only found in skeletal muscle cells and form a specific MyoD family of nuclear proteins that activate transcription. The members of the MyoD family are regulated by interactions with other proteins and by muscle electrical activity (Buckingham 1992, Dauncey & Gilmour 1996, Swatland 2004). In all probability, the gene expression regulating the specialisation of different fibre types in skeletal muscle is controlled by multiple transcription factors that act through combined mechanisms (Firulli & Olson 1997). An outline of the main events and regulatory factors involved in differentiation and maturation of a muscle fibre is given in Fig. 1.

![Diagram of myogenesis and differentiation](image)

**Fig. 1.** Summary of the main events and regulatory factors during myogenesis and differentiation of a skeletal muscle fibre. Positive regulators such as thyroid hormones, retinoids and insulin-like growth factors induce myoblasts to exit from the cell cycle. Many growth factors act in quite the opposite way by preventing cell cycle arrest and repressing myogenic gene expression. (modified from Dauncey & Gilmour 1996)

### 1.1.2 Morphogenesis

The onset of expression of myogenic proteins and the terminal differentiation of muscle fibres are regulated mainly by myogenin and MRF4 (Perry & Rudnick 2000). However, prior studies have shown that assembling the protein components in a myofibril is a
A complex event including many molecular reactions (Epstein & Fischman 1991). The contractile protein synthesis is regulated co-ordinately and the synthesis of each structural protein follows a unique pattern of regulation. The relative timing for appearance of mouse skeletal muscle proteins and structural components is illustrated in Fig. 2.

**Fig. 2.** The relative order of development in the skeletal muscle fibre of mouse. The stages in development are presented as days after fertilization. MHC; myosin heavy chain, MHCemb; embryonal isoform of MHC, MHCpn; perinatal isoform of MHC. (modified from Mänttäri & Järvilehto 2001)

The first of the proteins to be expressed and synthesized in developing muscle fibre is desmin, followed by titin. Muscle specific actin and myosin are detected next, and finally nebulin, troponin and tropomyosin are synthesized (Furst et al. 1989). The assembly of myofibrils proceeds with the formation of protein complexes, followed by accumulation of complexes into larger structures. The muscle fibre arrangement is a process regulated by protein-to-protein interactions. The plasma membrane and the components of submembranous cytoskeleton in particular are involved in protein organizing (Antin et al. 1981, Epstein & Fischman 1991).

The maturation of membrane systems of muscle fibre takes place simultaneously with the formation of myofilaments (Kelly 1971). After the formation of sarcolemma, two functionally interdependent membranous compartments, sarcoplasmic reticulum (SR) and transverse tubule (T-tubule) system start to assemble. The maturation proceeds in five stages: (1) increase in the amount of both SR and T-tubules, (2) increase in the number of
connections between SR and T-tubules, (3) gain of the typical adult shape of the membrane structures, (4) transverse orientation of the connective areas (triads) of SR and T-tubules, and (5) final differentiation of SR and T-tubule system (Franzini-Armstrong 1991). There are two main theories for the formation of T-tubules. According to Kelly (1971), early on in the development the initially formed peripheral couplings of SR and sarcolemma are swept into the cell and become part of the T-tubular system. On the other hand, Ezerman and Ishikawa (1967) noted intracellular combining of membrane vesicles and formation of primitive tubule-like structures. Most likely, T-tubule formation is a combination of the two models mentioned above (Flucher 1992).

1.1.3 Origin and diversity of muscle fibre types

During development each muscle fibre expresses a specific set of genes whose products determine its distinct contractile, metabolic and electrophysiological properties, i.e., the type of the fibre. The specialized properties of the fibres are imposed on them by temporal and spatial signals coming from tissues adjacent to somites (Kelly & Rubinstein 1994, Kronnie & Reggiani 2002). Due to the expression of multiple protein isoforms, a spectrum of functionally different fibres appears. Despite this, muscle fibres can be classified into similar phenotypes on the basis of various criteria, such as metabolic enzyme profiles and structural and functional properties (Pette & Staron 1993).

By comparing the identified muscle proteins and functional properties of the fibres it was found that these properties are closely related to the myosin heavy chain (MHC) isoforms (Schiaffino & Reggiani 1994). The sequence homology analysis of MHC isoforms reveals that slow MHC isoforms diverged from other MHCs in an ancient split. In contrast, the evolution of fast MHC isoforms was more parallel, since the isoforms within a vertebrate class are structurally quite similar (Lutz et al. 1998). In adult mammalian muscles, at least 11 MHC isoforms have been identified (Pette & Staron 2001). However, the multinucleate character of muscle fibres generates heterogeneity of protein expression within individual fibres, and hybrid fibres containing two or more MHC isoforms may develop (Newlands et al. 1998). Although determination of MHC isoform composition is one of the most suitable choices for classifying muscle fibres, many isoforms of other muscular proteins, for example myosin light chains and various Ca²⁺-regulatory proteins, are also expressed in specific fibres (Pette & Staron 2001).

The heterogeneity of skeletal muscle fibres and the molecular diversity explain the fact that the phenotypic profile of muscle cell is not considered definite. Muscle fibres are dynamic structures capable of adaptive changes under different conditions such as exercise training, altered innervation, and mechanical loading and unloading. Hormones and aging also have an impact on muscle fibre structural and functional properties (Larsson & Ansved 1995, Adams et al. 1999, Jakubiec-Puka et al. 1999, Loughna & Morgan 1999, Allen et al. 2001).
1.1.4 Structural basis of muscle contraction

The highly ordered structure of the skeletal muscle is an ideal example of a structure as a basis of function. The multinucleate muscle fibres of vertebrate are several millimetres long with a regular pattern of transverse stripes as observed under light and electron microscopy. Muscle fibres are composed of adjacent myofibrils with repeating units of sarcomeres. A sarcomere is considered the fundamental contractile element of the skeletal muscles.

1.1.4.1 Myofibril

A sarcomere is primarily made up of thick (myosin-containing) and thin (actin-containing) myofilaments precisely arranged within the myofibril. The actin filaments are anchored in Z-lines, which separate the sarcomeres. Within a sarcomere, actin filaments partially overlap with myosin filaments and form zones producing the characteristic bands of skeletal muscle. In addition to myosin, thick filaments also contain many additional proteins, whose functions are not well understood. Thin filaments, on the other hand, contain many well-known structural and regulatory proteins, such as nebulin, tropomyosin and subunits of troponin (Tn) complex, which take part in contraction of the fibre (Zot & Potter 1987). The highly ordered structure of muscle is probably assembled and stabilized by a giant muscle protein called titin. Titin is assumed to interact with C-protein and X-protein of the thick filament and to regulate the assembly of myosin. This hypothesis is also supported by the fact that titin appears early during development and thus controls the myofibril architecture (Trinick 1994).

1.1.4.2 The sarcoplasmic reticulum and the transverse tubules

In addition to the orderly array of muscle fibrils, the two intracellular membrane systems of skeletal muscle fibre, sarcoplasmic reticulum and transverse tubule system, also form an organized structure. The membrane of SR runs along the longitudinal axis of myofibrils and functions as a storage site for intracellular calcium (Stokes & Wagenknecht 2000). Under normal conditions, the stored Ca\(^{2+}\) within the SR is bound to a protein called calsequestrin (CLQ) (Franzini-Armstrong 1999). Calsequestrin is a linear polymer attached to the SR membrane by binding to intra-membrane proteins such as triadin, junctin and the ryanodine receptor (RyR), which is also known as the calcium release protein of SR. In addition to CLQ and RyR, the calcium ATPase or calcium pump protein (SERCA), responsible for pumping calcium from cytosol to lumen of SR was among the first proteins purified from the membrane of SR. Later studies have revealed that these three proteins are fibre-type specific and specialize in handling calcium in response to the needs of different fibre types (Sutko & Airey 1996, Beard et al. 2004).

The T-tubule system provides a structural bond between sarcolemma and the deeper portion of the fibre. The number of T-tubules varies among species and muscles. However, the distribution of tubules is similar within a given fibre. T-tubules consist of
free regions and junctional areas with SR. These specialized areas between the two membranes, also called couplings, contribute to the transmission of impulse from the depolarized T-tubules to the SR (Flucher 1992). In each triad, the T-tubule lies between two SR components, also called lateral sacs of the triad, or terminal cisternae of the SR (Fig. 3). If T-tubule forms a junction with only one SR cisterna, a dyad is formed (Franzini-Armstrong 1991). The proportion of junctional and non-junctional areas can vary between different muscle types (Delbono & Meissner 1996). The shape of transverse tubules is determined by the nature of the tubule. The non-junctional areas are typically small zigzag tubules with approximately round cross section. Tubules that participate in junctions are, on the other hand, straight and flattened (Franzini-Armstrong 1994).

Fig. 3. Schematic representation of a triad in mammalian skeletal muscle fibre illustrating the major components involved in excitation-contraction coupling. RyR, ryanodine receptor; DHPR, dihydropyridine receptor; SR, sarcoplasmic reticulum; T-tubule, transverse tubule; SERCA, sarcoplasmic reticulum Ca2+ pump.

1.1.4.3 Ultrastructure of the triad

The two membrane structures of the triad region are separated by a gap of 10-20 nm. The gap is bridged by so-called “foot” structures of ryanodine receptor molecules in the membrane of the terminal cisterna (Takeshima 1993). The feet form a tetragonal arrangement and where the junction is elongated, there are long parallel rows of feet in a
repetitive arrangement as visualized by Franzi-Armstrong (1973). As a whole, the RyR is comprised of two major components. In addition to the foot region of the protein, a larger multidomain assembly embedded in the membrane of SR gives the molecule a square prism-like outlook.

A second component of the triad, calsequestrin, is expressed in the lumen of the terminal cisternae of SR. There are two isoforms of CLQ in muscle tissue. The skeletal isoform is expressed in fast-twitch skeletal muscle, while the cardiac isoform is found in the heart and in slow-twitch skeletal muscle. The amino acid sequence of the CLQ protein is highly conserved between the isoforms and also between different species (Fliegel et al. 1990, Beard et al. 2004).

Directly across the terminal regions of RyRs, four dihydropyridine receptor (DHPR) molecules (a structure called tetrad) are located in exact correspondence to the four subunits of the foot structure. In the junctional gap, every other foot in the SR membrane is adjacent to a tetrad of DHPRs. The tetrad/RyR relation of 1:2 is the most common one, but lower and higher ratios have, however, been described (Franzini-Armstrong & Jorgensen 1994). The feet with no association with tetrads form peripheral couplings with sarcolemma or occur independently from the complete assembly of junctional T-tubules (Flucher 1992).

Recently, the techniques in studying the ultrastructure of the triads have brought new insights into the membrane assembly. Wagenknecht and co-authors (2002) described for the first time the assembly of components in triad junctions by cryoelectron tomography. One difference to the previous studies was the orientation of the triadic gap area. Contrary to the former hypotheses, there appears to be a gap of several nm in width between the RyR and T-tubule membrane. However, some of the feet of RyRs appear to be connected to T-tubule membrane via bridging dense material that is assumed to correspond to domains of the DHPRs. Thus there is a direct contact between RyR and DHPR at the triad. In addition to the structure of the triad junction, there are also differences in the arrangement of RyRs compared to the images obtained by using electron microscopy techniques. As visualized with electron tomography, the array of RyRs seems to be more irregular than previously described.

Mdg allele when homozygous in mice leads to the absence of DHPRs in muscle, and to a disease called muscular dysgenesis (Chaudhari 1992). Furthermore, dysgenic muscle seems to lack triads with clearly visible feet, although some triads with normal disposition of feet and CLQ could be found. Based on the fact that ultrastructurally normal triads can be formed in cells not expressing DHPRs, it has been proposed that the DHPR is not required for the assembly of triad and that the absence of the expression of the receptor protein is not critical for the appearance of triads (Franzini-Armstrong et al. 1991). These previous results suggest that there have to be proteins maintaining the structure of a triad even though RyR or DHPR is absent. And in fact, two additional proteins present in the triad area are presumably employed in the formation or maintenance of the membrane junctions. The proteins belong to a conserved family of membrane complex proteins and are named mitsugumin and junctophilin (Takeshima et al. 2000).
1.1.5 Muscle contraction

Contraction of a muscle is result of the relative sliding of thick and thin filaments past each other. Muscle work and motion arises from the action of the cross-bridges composed of the subfragment 1 (S1) portion of myosin molecule. The cross-bridges interact independently with the thin filaments so that the actin filaments are pulled towards the centre of sarcomere. Since the actin filaments are anchored in the Z-disks, the sarcomere length is decreased. Mg-ATP serves as the direct source of energy for muscle contraction. The S1 portion of myosin molecule catalyses the hydrolysis of ATP to ADP and inorganic phosphate. As a result of the hydrolysis, myosin head binds to and rotates against actin filament.

1.1.5.1 Excitation-contraction coupling

The activation of cross-bridge attachment and force production is controlled by the change in the membrane potential. The action potential propagating along the axon of a motoneuron activates release of acetylcholine through the terminal membrane of the neuron. At the neuromuscular junction the acetylcholine binds to postsynaptic receptor proteins opening ion channels in muscle fibre sarcolemma where depolarization begins and proceeds as the first phase of excitation-contraction (E-C) coupling. The coupling between excitation and contraction depends on the calcium ion release from the SR.

In striated muscle fibre Ca\(^{2+}\) binds to troponin C, which is one of the three globular subunits of the Tn complex on the actin filament. The binding changes the conformation of the Tn in such a way that the tropomyosin strands attached to Tn shift relative to the actin strand and thus expose the myosin-binding sites, allowing actin and myosin to interact.

The first study indicating that the calcium release was a consequence of intramembrane charge movement came from Schneider and Chandler (1973). They described large groups of molecules in the junctional area of T-tubules and SR. They also observed that the molecules embedded in the T-tubular membrane changed conformation as a response to the change in the membrane potential. Later Chandler and co-authors (1976) described an extended mechanical link model of E-C coupling where the conformation change of the charged particle mechanically controls the release of Ca\(^{2+}\) from SR. Rios and Brum (1987) were the first to report that the charged particle responsible for the transduction of charge movement to calcium release is the dihydropyridine receptor molecule. It has since been shown that (1) there is a direct link between DHPRs and ryanodine receptors (Block et al. 1988), (2) E-C coupling in skeletal muscle depends upon a functional interaction between DHPRs and RyRs (Fleischer & Inui 1989) and that (3) the coupling is bi-directional; i.e., DHPRs receive a retrograde, current enhancing signal from RyRs (Nakai et al. 1996, Grabner et al. 1999).

During E-C coupling, calsequestrin also undergoes a conformation change before Ca\(^{2+}\) release. This structural change, in addition to that upon calcium binding, suggests that the impulse is transmitted from T-tubule membrane to the calcium binding protein.
Furthermore, this indicates that CLQ is in close contact with ryanodine receptor and that it also regulates the function of RyR (Ikemoto et al. 1991).

Quite recently, two additional mechanisms have been introduced at the side of the mechanical-link concept described above. The first of these states that the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), i.e., the influx of calcium through calcium channels in the T-tubule membrane, induces an increase in calcium concentration level in the myoplasm (Ohta et al. 1989). The increase in myoplasmic Ca\(^{2+}\) concentration causes further release of Ca\(^{2+}\) from SR and thus makes CICR important especially in the heart muscle (Fabiato 1983). In skeletal muscle, however, the calcium release from SR can also be induced in the absence of calcium influx (Gonzales-Serratos & Valle-Aguilera 1982). It has been suggested that the calcium current is needed to maintain the calcium stores inside the fibre (Beam et al. 1989).

The third coupling mechanism is based on an intracellular messenger system. As a result of depolarization, phosphodiesterase located in the T-tubule membrane is activated. The enzyme hydrolyses phosphatidylinositol (1,4,5)-trisphosphate (InsP\(_3\)) from its precursor phosphatidylinositol (4,5)-bisphosphate (PIP\(_2\)). The released InsP\(_3\) molecules bind to the calcium release channel in SR and a passive ion flow to the myoplasm begins (Talon et al. 1999). In skeletal muscle the concentration of PIP\(_2\) is probably too low to play a direct role in the coupling process (Milting et al. 1994). In smooth muscle, however, the signal transmission is quite effective (del Valle-Rodriguez et al. 2003).

### 1.2 Voltage-gated sodium channels in skeletal muscle

Voltage-gated sodium channels play a critical role in controlling the electrical excitability of animal cells, being primarily responsible for the depolarizing phase of action potential. In skeletal muscle fibre, Na\(^+\) channels are found in both the surface and T-tubular membranes with a well-defined distribution (Caldwell 1986, Haimovich et al. 1987).

The Na\(^+\) channel isolated from rat and rabbit skeletal muscle plasma membrane contains a large α subunit of 260 kDa and a small β subunit of approximately 38 kDa. The β subunit is noncovalently associated with α subunit in a 1:1 stoichiometry. The pore forming α subunit contains structural similarities with that of voltage-dependent calcium and potassium channels, which has led to the notion that they belong to a superfamily having a basic design (Barchi 1986).

Mammalian skeletal muscle expresses two forms of Na\(^+\) channel. The Na\(^+\) channel in adult skeletal muscle (designated SkM1 or NaV1.4) is tetrodoxin (TTX) sensitive, while that in embryonic and cardiac muscle (SkM2 or NaV1.5) is relatively insensitive to TTX (Barchi 1995). According to Zhou and co-authors (1991), the conductance of NaV1.4 is approximately three times higher as compared to the conductance of NaV1.5. The individual NaV1.4 channels switch between two principal gating modes, operating either only once per depolarization, or repeatedly in long bursts. It appears that the equilibrium among gating modes is regulated by a modulating factor encoded in adult skeletal muscle.

The conductance of a single Na\(^+\) channel has been estimated by comparing the binding of radiolabelled toxins with macroscopic current measurements, and by measuring the
current through single channels using the patch-clamp technique. Based on electrophysiological data, one of the characteristics of sodium channels is the steep voltage dependence of activation. This requires movement of voltage sensors (charged amino acid residues) under the force of electric field leading to a conformational change in the channel protein. In addition to voltage dependency, fast inactivation is also typical for skeletal muscle sodium channel. Na$^+$ channels inactivate within a few milliseconds of opening and remain unavailable for opening by a subsequent depolarization until they have recovered from the inactivation (Catterall 2000a).

1.3 Dihydropyridine sensitive calcium channels in skeletal muscle

Calcium ions are an important part of the physiology of a muscle cell. They regulate the functions of a muscle and are essential for cellular contraction. Most of the calcium flow of muscle cells occurs through ion channels and this flow is voltage-dependent. The voltage-sensitive calcium channels of striated skeletal muscle tissue are called slow calcium channels, because they need a potent stimulus to be activated and they close slowly (Bean 1989). The channel mediates long-lasting calcium current that is activated when the membrane potential changes (Cognard et al. 1986). The slow calcium channel of a muscle cell belongs to L-type (long lasting) channels that are found in almost all tissue types. L-type calcium channels are slowly inactivated, dihydropyridine (DHP)-sensitive ion channels with a high threshold value (Bean 1989). DHP and its derivatives, phenylalkylamines (verapamil) and bentsodiazepines (diltiazem), reduce the permeability of the channel when they bind to the receptor (Catterall et al. 1989).

The L-type calcium channel, first solubilized and purified from the T-tubule membranes of skeletal muscle, is a heteromultimer composed of five subunits in 1:1:1:1:1 molar ratio (Fig. 4). The largest subunits are $\alpha_1$ (175-212 kD), $\alpha_2$ (140 kD), $\beta$ (55 kD), and the $\gamma$ (33 kD). A small polypeptide chain, the $\delta$ subunit (24-33 kD), is connected to the $\alpha_2$ subunit by a disulphide link. The $\delta$ subunit forms a complex $\alpha_2/\delta$ with the $\alpha_2$ (Mori et al. 1993). The $\beta$ subunit is located peripherally inside the membrane (Catterall 1993). The $\alpha_2$, $\beta$, $\gamma$ and $\delta$ subunits of the channel protein complex regulate the functions of the $\alpha_1$ subunit. The $\beta$ subunit in particular regulates activation and inactivation kinetics by influencing the number and location of DHP receptors. The $\gamma$ subunit has been found to increase the inactivation speed of a channel, although the role of the subunit is not well understood. The $\delta$ subunit regulates voltage-sensitive activation, and the $\alpha_2$ subunit provides structural support and has an influence on the calcium flow through the channel (Gutierrez et al. 1991, Singer et al. 1991).
Fig. 4. Subunit structure of the voltage-gated L-type calcium channel. The primary structures of the subunits are illustrated as transmembrane folding diagrams of the $\alpha_1$, $\alpha_2\delta$ and $\gamma$ subunits. The $\beta$ subunit is located in the cytosol. Cylinders represent $\alpha$-helical segments. (modified from Catteral 1993)

1.3.1 Dihydropyridine receptor

1.3.1.1 Structure and molecular pharmacology

The dihydropyridine receptor is located in the $\alpha_1$ subunit of the L-type calcium channel in the skeletal muscle T-tubule membrane. The subunit also contains regulatory areas essential to the functioning of the channel. In mammalian cells, the $\alpha_1$ subunit can alone function as a voltage-sensitive ion channel. The subunit consists of 1873 amino acid residues with a molecular mass of either 175 kDa or 212 kDa. The 212-kDa form contains the full sequence encoded by the mRNA, while the 175-kDa form lacks the C-terminal amino acids (Tanabe et al. 1987). The transmembrane structure of the protein resembles the corresponding pore-forming $\alpha$ subunit of sodium channel, suggesting that the genes encoding these two receptors arose from a common ancestor. The amino acid sequence of the DHPR is organized in four repetitive domains (domains I-IV, Fig. 4), each of which contains six transmembrane segments (S1-S6). Five of these segments (S1, S2, S3, S5 and S6) are hydrophobic and one (S4) positively charged. The segments are formed of $\alpha$ helical domains arranged symmetrically around the channel structure. The positively charged S4 segments serve as voltage sensors. Under the influence of electric field, the S4 segments move outward and rotate initiating a conformational change of the
channel protein and thus opening the pore (Catterall 2000b). Prior works of Tanabe and co-authors (1990) and Nakai and co-authors (1998) revealed regions in the II-III loop critical for excitation-contraction coupling. The region specific for skeletal muscle tissue also indicates an important determinant of skeletal type E-C coupling.

The $\alpha_1$ subunit contains three separate, allosterically linked binding sites for the three main classes of calcium antagonists. Each of the three types of channel blockers interacts with specific receptor site and inhibits the influx of extracellular calcium through the channel. Hence, the blockers are often used in clinical practice. Of the three binding sites, the 1,4-dihydropyridine receptor is the most accessible since the location of the receptor is on the surface of the channel. Receptors for phenylalkylamines and benzothiazepines are also located on the $\alpha_1$ subunit of the channel. The three receptor sites exhibit complex interaction. Therefore, antagonists binding to the dihydropyridine receptor may increase the affinity of other binding molecules, and vice versa. On the other hand, some 1,4-dihydropyridines may also have inhibiting effects at higher dosages (Triggle 1992). The maximum specific DHP binding capacity of transverse tubules isolated from skeletal muscle tissue is about 15-80 pmol/mg protein (Catterall 1988).

1.3.1.2 Dual function of the dihydropyridine receptor

The dihydropyridine receptor has two functions: on the one hand it functions as a voltage-sensitive protein controlling mechanically the release of calcium from the sarcoplasmic reticulum, and on the other hand as an ion channel for slow calcium current between the extra- and intracellular spaces. Blocking the ion flow by using cadmium does not, however, have an impact on the contraction of a muscle (Grabner et al. 1991). The kinetics of these two functional mechanisms differ greatly from each other. It takes less than 5 ms from the depolarization of the sarcolemma to the release of calcium from the sarcoplasmic reticulum, whereas the initiation of the calcium flow through the T-tubular membrane takes more than 100 ms (Ma et al. 1996). Besides, the potential threshold for SR calcium release is about 30 mV more negative than the threshold for the activation of calcium current through the channel molecule (Pizarro et al. 1988). The ability of the L-type calcium channel to conduct ions does not seem to be of great significance to the functioning of the muscles of an adult mammal. First, the ion current is activated too slowly to generate contraction of the fibre. Second, the contraction continues even if the extracellular calcium is removed, and finally, blocking the channel does not prevent contraction (Gonzalezs-Serratos & Valle-Aguilera 1982). Nevertheless, a notable increase in the magnitude of inward calcium current has been noted in the developing muscle, thus implicating an important role of the current in maturation (Beam et al. 1989). It has also been shown that the contribution of the ion flow to E-C coupling diminishes during development (Cognard et al. 1992). There is a clear distinction between the skeletal-muscle-type and the heart-type E-C coupling. In myocardium, the ion flow is important for the contraction of the muscle (Martonosi 1994).

The dual functions of DHPR can be influenced separately. For example, a DHPR specific blocker can simultaneously block the Ca$^{2+}$ current while, on the other hand, it induces Ca$^{2+}$ release from the SR. DHPs can thus have both stimulatory and inhibitory
effects on E-C coupling (Weigl et al. 2000). Furthermore, it has been suggested that most of the DHPR receptors are unable to function as calcium channels, but serve a different function, probably initiating calcium release from the SR. This hypothesis is supported by the fact that only about five percent of the DHPRs are active in ion conductance either in skeletal muscle preparations in vivo or in purified T-tubule preparations (Schwartz et al. 1985). It has therefore been suggested that the 212-kDa form of DHPR is specialised in calcium conducting while the 175-kDa form lacking the C-terminal tail is the form serving as the voltage sensor in E-C coupling. The density of the forms also supports this finding, since the 212-kDa form represents only five percent of the DHPRs while the lighter form is much more abundant. Thus, the calcium conductance may be associated with the structure of DHPR, and the structure inducing the ion flow through the channel protein is located in the C-terminal tail portion of the DHPR (De Jongh et al. 1989).

1.3.1.3 DHPR adaptation

Previous findings suggest that the ontogeny of DHPRs during myogenesis may be controlled similarly as that of other muscle-specific gene products (Schneider & Olson 1988). It has been shown that the DHPR mRNA expression is both tissue-specific and developmentally regulated in mouse skeletal muscle. It has also been observed that the expression of DHPR mRNA is more abundant in fast as opposed to slow muscle. Furthermore, DHPR mRNA and protein expression are induced as late events during myogenic differentiation (Shih et al. 1990). Although previous results show that there are quantitative differences in slow Ca\(^{2+}\) channel mRNA expression during development, the studies on the developmental expression of DHPR protein are few in number.

In addition to the developmental signals influencing the expression of DHPRs, also some physiological signals affect the genes encoding the DHPR protein. For example, it has been observed that DHPRs of skeletal muscle are downregulated by long-term depolarization (Escamilla et al. 2001). This indicates that the decrease in the expression of the L-type calcium channel might have a protective role in preventing the muscle from calcium overload in the long term. On the contrary, a long-term blockade of DHPR induces upregulation of the receptor protein (García et al. 1999).

Previous studies have indicated that chronic low-frequency stimulation triggers decline in the level of DHPR (Ohlendieck et al. 1999). Moreover, it has been noticed that the levels of other E-C coupling elements, such as RyR, SERCA, and CLQ, follow a similar kind of pattern.

The impact of different physiological conditions on DHPR expression has been demonstrated in some studies. For example, evidence that alterations in DHPR/RyR coupling are major determinants of E-C uncoupling in aged skeletal muscles has been published (Renganathan et al. 1997). Furthermore, the peak intracellular Ca\(^{2+}\) resulting from the depolarization of plasma membrane has been shown to be significantly smaller in muscle fibres from old mice than in fibres from middle-aged or young mice (Wang et al. 2000). However, no published data on the direct effect of aging on the DHPR expression were found. Some hormones also have profound influence on the DHPR
density in skeletal muscle T-tubules. One example is the insulin-like growth factor that prevents the age-related decline in DHPR expression (Renganathan et al. 1998).

A few studies have investigated the effect of increased muscular activity on DHPR gene expression. DHPR protein content is shown to increase as a result of endurance training in rat muscles (Saborido et al. 1995). However, the contribution of the regulated gene expression to this adaptation is not known. Although many factors affect the DHPR level, the gene expression is relatively insensitive to denervation (Shih et al. 1990).

Taken together, previous results strongly suggest that biochemical alterations reflect myofibrillar protein adaptations and that there are muscle fibre-type specific differences in the fine-tuning of the E-C coupling. Of the main E-C coupling-relaxing cycle components, RyR, SERCA and CLQ all have many isoforms resulting in different fibre phenotypes (Sutko & Airey 1996, Beard et al. 2004). Although parallel changes have been observed between the components of E-C coupling as a response to adaptation to different physiological signals, the fibre-type specificity of DHPR has remained an open question.
2 Aims of the study

It is well established that voltage-sensitive ion channels selective for calcium ions have an important physiological role in muscle cell signalling. The essential link between changes in membrane potential and initiation of contraction is the dihydropyridine receptor acting as a voltage sensor for intracellular calcium release and as an ion-conducting channel. Although the molecular properties and behaviour of dihydropyridine receptor have been investigated quite intensively, very little is known about the receptor characteristics. In this work, the expression of DHPR was examined from the developmental and strain physiological point of view in two vertebrate taxa, mammals and fish. The specific objectives of this study were:

1. to evaluate the expression of dihydropyridine receptor during postnatal development and to relate the build-up to the maturation of the muscle fibre;
2. to define the precise cellular distribution of the dihydropyridine receptor in order to characterize the excitation-contraction coupling properties of different fibre types and different muscles;
3. to study the adaptive response of excitation-contraction coupling machinery to increased muscular activity in laboratory as well as in natural environment of the animal;
4. to analyse the expression of dihydropyridine receptor in mammalian as well as in fish muscle in order to gain a comparative aspect to protein expression.
3 Material and methods

3.1 Animals

The experiments were performed on skeletal muscles of mouse (*Mus musculus*, random strain) (I-IV) and Atlantic salmon (*Salmo salar*) (V). The age of the mice varied from new-born to 63-day-old depending on the purpose of the experiment. In salmon, the parr-smolt transformation had been completed, and the fish were 2 years old. Mice were obtained from the Laboratory Animal Centre of the University of Oulu, and salmon from Finnish Game and Fisheries Research Institute (Taivalkoski, Finland).

The maintenance of all the animals used is described in detail in the original papers (I-V). All the treatments of experimental animals were reviewed and accepted by the Committee of Animal Experimentation at the University of Oulu (licences 056/01, 02/03, 046/03, 029/03 and 100/03).

3.2 Electrophysiological measurements

3.2.1 Conduction velocity measurements

The conduction velocity in *m. sartorius* was measured as previously described (JärviLehto & Rissanen 1987). Briefly, the responses of the electrical stimulation at two locations on the muscle surface were recorded and the time difference of the two corresponding records was calculated.

3.2.2 Force measurements

Adult mice (23-45 g) were killed by paracervical dislocation. *M. gastrocnemius* (GAS), *m. rectus femoris* (RF) and *m. gluteus maximus* (GLU) were dissected out and positioned
between a fixed clamp at the base of an organ bath and a Grass FTO3C force-
displacement transducer. The muscles were maintained at 37°C in a bath containing 
oxygenated Ringer solution of the following millimolar compositions: NaCl, 137.0; 
CaCl₂, 1.8; KCl, 2.7; MgSO₄·7H₂O, 1.0; NaH₂PO₄, 0.4; NaHCO₃, 12.0 and glucose 5.5. 
The muscles were equilibrated for 2 min and supramaximally stimulated using steel 
electrodes, with pulses of 1 ms duration at 80 V. The muscle length was adjusted to elicit 
maximal twitch force. Recordings of transducer output after three impulses per muscle 
were A/D converted and collected on a PC at a sample frequency of 1000 Hz. 

After measuring the maximal twitch force of a muscle, the Ringer solution was 
replaced with a solution containing 1µM nifedipine (Sigma-Aldrich, St. Louis, MO) 
prepared as a concentrated stock solution in DMSO and protected from light. The force 
measurement was assessed as described above. Furthermore, a series of measurements 
was made using nifedipine in a concentration range of 2-30 µM. To exclude the 
possibility of fatigue caused by successive stimulations, the protocol control 
measurement was performed similarly devoid of nifedipine.

3.3 Endurance training

Mice were adapted to an inverse 12:12 h light-dark cycle (dark period from 9.00 to 
21.00) before beginning of the exercise regimen for the convenience of the experiments. 
The exercising animals ran on a motor-driven treadmill and performed five running 
Sessions a week. The first running session at a speed of 22 m/min and with a 10° slope 
lasted 5 min. After 4 weeks, the running time was prolonged to 10 min. Finally, after 10 
weeks, the mice completed sessions of 20 min running for 10 days. Since the mice were 
unable to run in the darkness, a dim light was used during the running sessions.

3.4 Laboratory assays

3.4.1 Histological techniques

3.4.1.1 Paraffin sections and EM

Muscle samples were collected from mice of different age and fixed for 90 min in 
Bouin’s solution, then dehydrated, embedded in paraffin and cut into 6 µm thick sections 
as previously described by Bancroft and Stevens (1990). The sections were stained with 
allochrome method (Lillie 1951) and viewed with light microscope (Polyvar, Reichert-
Jung, Austria).

Small specimens (~ 1 mm³) were cut from the muscle tissue samples and incubated in the 
prefixation solution (4% formaldehyde/1% glutaraldehyde in 0.1M phosphate buffer), 
washed in phosphate buffer solution, dehydrated and postfixed in osmium tetroxide. The 
tissue samples were then embedded in Epon. Thin sections were made and double-stained
with uranyl acetate and Reynold’s lead citrate (Reynolds 1963). Samples were examined with a Jeol JEM-100CXII (Japan) electron microscope.

### 3.4.1.2 Fluorescence techniques

For the DHPR expression studies, RF and GAS muscles from the hind limb of mouse were dissected out and rapidly frozen in liquid nitrogen. Tissue samples were cut into 8-10 µm thick cryosections and incubated in 15 (I) or 20 (III, IV) nM high affinity (-) enantiomer of dihydropyridine labelled with orange fluorophore for 90 min. After washes, the sections were covered with antifade reagent, viewed and photographed with photomicroscope (I) or confocal laser scanning microscope (III, IV) by using excitation at 546 nm. Control sections were incubated in phosphate buffer devoid of dihydropyridine. A control with cold, non-fluorescent DHP was also performed according to Larsson and co-authors (1998) by preincubating the samples with 10 µM nifedipine in phosphate buffer for 10 min prior to the addition of dihydropyridine conjugate.

In fish migration experiment (V), blocks of red and white muscles were taken between adipose fin and tail. The sections were cut at a thickness of 14 µm and incubated in 20 nM dihydropyridine solution. Otherwise the treatment was like that described above.

### 3.4.1.3 Enzyme histochemistry

Tissue samples for histological analyses were collected from RF, GAS (I, III) and GLU (III) muscles immediately after paracervical dislocation, frozen with liquid nitrogen and sectioned (10 µm). The sections were stained histochemically for the activity of succinate dehydrogenase (SDH) as described by Nachlas and co-authors (1957) and for myosin ATPase at sodium barbital buffer (pH 9.4) after pre-incubation at either pH 4.3, 4.6 or 10.3 according to Brooke and Kaiser (1970) (I) or Hämäläinen and Pette (1993) (III). Fibres were classified into either fast glycolytic (FG), fast oxidative glycolytic (FOG) and slow oxidative (SO) (I) or I, IIA, IIB, and IID (III) type according to their metabolic properties and the method used. Cross-sectional areas and diameters of different fibre types were measured with a Micro Scale TM/TC image analyser.

The staining of motor endplates on the muscle fibre plasma membrane was based on the histochemical method for acetylcholinesterase (Karnovsky & Roots 1964). The substrate buffer used in this study was sodium acetate buffer, pH 6.0.

### 3.4.1.4 Immunohistochemistry

In order to study the expression of sodium channel during postnatal development, muscle tissue samples from mice at different age were collected, cut with a cryostat microtome into 10 µm thick sections and treated as follows: wash with 0.1M phosphate buffer (PB), followed by 0.3% TritonX-100/10% BSA in PB wash, overnight incubation in the monoclonal antibody solution (0.7 µg/ml), salt wash in 2.7% NaCl solution and a final
wash with PB. After the salt wash, the samples were incubated in Alexa Fluor – conjugated secondary antibody for 60 min, rinsed with 0.3% TritonX-100/10% BSA in PB, 0.1M PB and 0.05 M PB, and covered with antifade reagent. The sections were viewed with confocal laser scanning microscope by using excitation at 488 nm.

The samples were also stained with an enzyme-label detection method by using a polyclonal primary antibody (Anti-Pan Sodium channel) and an alkaline phosphatase – conjugated secondary antibody. The sodium channel protein detected by the antibody was visualized with BCIP/NBT substrate buffer incubation.

### 3.4.2 SDS-polyacrylamide gel electrophoresis and Western blotting

The SDS-polyacrylamide gel electrophoreses (SDS-PAGEs) were performed in a Protean II xi electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) under reducing conditions (Laemmli 1970) using a 3.1% sodium dodecyl sulphate polyacrylamide stacking gel and a 5-8% gradient gel. The protein concentration in the samples was measured with a protein assay kit (Bio-Rad) according to the method of Bradford (1976). The protein bands were stained with Coomassie brilliant blue. The Western blot analysis was applied on myosin heavy chain isoforms (V) and dihydropyridine receptor (IV) as described (Towbin et al. 1979). The proteins were electrophoretically transferred to pure nitrocellulose membrane (Bio-Rad), and the blots were first incubated with a monoclonal primary antibody, then with a secondary antibody.

**Table 1. List of antibodies used**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Used in</th>
</tr>
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<tbody>
<tr>
<td>Primary antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-pan Na⁺ channel</td>
<td>Alomone Labs</td>
<td>II</td>
</tr>
<tr>
<td>Anti-Sodium channel (Pan)</td>
<td>Sigma-Aldrich Inc</td>
<td>II</td>
</tr>
<tr>
<td>L-type Ca²⁺ CP α1S mAb</td>
<td>Santa Cruz Biotechnology</td>
<td>IV</td>
</tr>
<tr>
<td>MHCf mouse mAb</td>
<td>Novocastra Laboratories Ltd</td>
<td>V</td>
</tr>
<tr>
<td>MHCs mouse mAb</td>
<td>Novocastra Laboratories Ltd</td>
<td>V</td>
</tr>
<tr>
<td>MHCf mouse mAb</td>
<td>DPC Biermann</td>
<td>V</td>
</tr>
<tr>
<td>Secondary antibody</td>
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<td></td>
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<tr>
<td>AP conjugated IgG, goat anti rabbit</td>
<td>Bio-Rad</td>
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<tr>
<td>Alexa Fluor 488, goat anti mouse IgG</td>
<td>Molecular Probes</td>
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</tr>
<tr>
<td>AP conjugated IgG, goat anti mouse</td>
<td>Bio-Rad</td>
<td>IV, V</td>
</tr>
</tbody>
</table>

The densitograms of the electrophoretic protein patterns and protein bands in membranes were produced and analysed by the FluorS MultiImager program (Bio-Rad).
3.4.3 **RT-PCR**

For the RT-PCR analysis, total RNA from muscles of trained and control mice was isolated with the QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech, UK). In brief, Cacna1S mRNA levels for mouse samples were measured by quantitative reverse transcription-PCR analysis as described by Majalahti-Palviainen and co-authors (2000). Forward and reverse primers for mRNA detection were 5’-CGAGGAGGTTGGCCAGG-3’ and 5’-GCATAGAGAAGCCAAAGTTGTCG-3’, respectively. Cacna1S amplicon was detected using a fluorogenic probe 5’-Fam-CCCAACCACCGCATACCCACT-Tamra-3’. The primers and the probe for the 18S amplicon were 5’-TGGTTGCAAAGCTGAAACTTAAAG-3’, 5’-AGTCAAATTAAGCCCGCAGGC-3’ and 5’-Vic-CCTGGGTGGTCCCCCTCGTCA-Tamra-3’, respectively.

3.5 **Statistical analysis**

Statistical analysis was performed by using the SPSS software for Windows software. The values of $p < 0.05$ were considered significant. The values were presented as means ± standard error (SE).

**Study I:** The cross-sectional area and fibre diameter of 10 fibres of each histochemically identified fibre type was measured with an image analyser. The procedure was also carried out for ten fibres labelled with ST-BODIPY. The possible correlations in all analysed parameters between identified fibre types and fibres exhibiting different fluorescence intensities were revealed with a t-test for equality of means.

**Study II:** The relationships between the studied variants (age, conduction velocity and sodium channel density) were examined using Spearman’s nonparametric correlation coefficient.

**Study III:** The statistical significance of differences between means of the contraction forces measured before and after the addition of the calcium channel blocker nifedipine was evaluated by paired samples t-test. The significance of differences between the muscles was evaluated with one-way analysis of variance. The differences in fibre type composition between the three muscles studied were analysed with independent samples t-test for Equality of Means.

**Study IV:** The DHPR densities between the control and trained group of mice were measured and the statistical differences between means were evaluated by Student’s t-test. The relationship between DHPR density and MHC isoform was determined using Pearson’s parametric correlation coefficient.

**Study V:** The difference between the average intensities in SDH-stained samples from salmon muscles was statistically analysed with Student’s t-test.
4 Results

In order to elucidate the expression and properties of dihydropyridine receptor associated with postnatal development of skeletal muscle, a series of experiments was performed as previously described. This chapter will summarize the results obtained. The complete set of results is presented in the original papers.

4.1 Developmental changes

*Structural development.* The structure of muscle tissue in one-day-old mice is not fully developed. The individual fibres inside the epimysium are rather loosely organized, and do not make up dense fibre bundles (Fig. 5a). After six postnatal days, the muscle fibres are already arranged in parallel arrays and form a more dense structure (Fig. 5b). In the muscle of a 15-day-old mouse, a large number of dividing fibroblasts can still be seen. This is indicative of a large proportion of interfibrous connective tissue. At this age, the striation becomes visible (Fig. 5c). The muscle tissue of an adult mouse is compact, consisting of parallel fibres and several tight junctions between them (Fig. 5d).

Studies with electron microscopy also reveal the undeveloped structure of muscle in one-day-old mouse (Fig. 6a). The myofibrils are surrounded by an intracellular matrix and the general impression of the tissue is highly irregular. During maturation, the tissue becomes more organized and compact: detectable membrane structures can be seen for the first time after six postnatal days, and the relative proportion of cytosol is decreased (Fig. 6b). After 9 days, the Z-line and all the zones of the sarcomere are regularly arranged (Fig. 6c). In the samples from 15-day-old mice the muscle tissue is already dense and organized (Fig. 6d).
According to the results, the membrane structures develop further postnatally. The intramembranous compartments are irregularly organized in the samples taken right after birth, whereas in the samples of older mice regular structures are formed. The triads, located at the A-I junction and composed of T-tubule and cisternae of sarcoplasmic reticulum, are detectable for the first time in the one-day-old mouse. This appearance is, however, only occasional at this age. In the muscle of 15-day-old mouse, the triads are more abundant and clearly seen (Fig. 7a). In the muscle of adult mouse, the triad structures are orderly arranged (Fig. 7b).

**DHPRs in developing muscle.** The changes in the density of dihydropyridine receptors after birth were analysed using a fluorophore-conjugated dihydropyridine molecule selectively binding to the DHPR. Fluorescence was not detected either in the samples from new-born mice or in the controls (I). The dihydropyridines are clearly detectable in the samples of 21-day-old mice, especially at the edges of the fibres. The intensity of this fluorescence is however moderate when compared to that in muscles of adult mice, where DHPRs occur in large numbers.

**Sodium channels in developing muscle.** In order to see how the fundamental maturation of the muscle and the expression of DHPR are related, the expression of sodium channels and the conduction velocity of the plasma membrane of the muscle were
also determined (II). Sodium channels, determined with an immunohistochemical method, are not seen until at the age of 8 days. Labelling is intensified along with aging and attains a remarkable increase in the samples from 25- to 30-day-old mice. Furthermore, the staining is not homogenous on the surface membrane of the fibres in the samples of older mice, but aggregates in repeated areas on the membrane. The comparison of aggregation of sodium channel antigens and motor endplates reveals high sodium channel densities near the neuromuscular junctions.

The conduction velocity of skeletal muscle plasma membrane is clearly increased as a function of age; however, a slight decrease is seen at the age of 8 to 13 days. Spearman’s correlation analysis indicates a significant correlation between age and conduction velocity (P < 0.05, II).

Fig. 6. Electron micrographs of *m. rectus femoris* from one (a), six (b), nine (c), and fifteen (d) days old mice. Bar 0.5 µm.
4.2 Fibre type specificity of dihydropyridine receptor

The intensity of fluorescence is not evenly distributed in all fibres labelled with a fluorophore-conjugated DHP (Fig. 8). By using enzyme histochemical staining, three different fibre types are detected in the cryosections of the muscles studied (1). Fibres with intensive fluorescence and histochemically identified fibre types were combined
with image analysis. The cross sectional area and the diameter of the fibre type with the highest fluorescence intensity differ significantly from the values of fast glycolytic (FG) and slow oxidative (SO) fibres (I, Table I). The difference with fast oxidative glycolytic (FOG) fibre is, however, non-significant, suggesting that the fibres with strong fluorescence intensity belong to the FOG type.

Fig. 8. Histological localisation of DHPR in mouse skeletal muscle. A cryostat section of *m. rectus femoris* was stained with fluorophore conjugated high affinity (-)-enantiomer of dihydropyridine. Bar 15 µm.

In order to correlate the DHPR expression with a more specific muscle fibre typing, the histochemical procedure was repeated using three different pHs instead of two. On the basis of the staining intensity, the muscle fibres strongly expressing DHPRs correspond to the IIA fibre type (III, Fig. 2).

To study the function of DHPRs in muscles which differ in regard to their fibre type composition, the effect of DHPR blocker on single twitch forces of the muscles was measured (III). The main fibre type in GLU and RF, determined with mATPase staining, is IIA (45.0 and 38.1%, respectively). In GAS the major fibre type is IID (51.5%), while the proportion of IIA fibre type is considerably lower (22.7%). The DHP blocker was inhibitory in all the muscles studied. Force production, determined as the maximum contraction force in proportion to the cross-sectional area of the muscle, was reduced in GLU and RF by 28.3 ± 3.5% and 27.6 ± 3.2%, respectively. The weakening was
significantly (P = 0.023) higher in GLU and RF as compared with GAS (16.1 ± 2.5%).
The results are presented in III, Table I; III, Fig. 3; thesis, Fig. 9.

Fig. 9. The remaining force (%) of GAS, RF and GLU after addition of blocker solution. The
significance of the differences between the muscles in attenuation of the contraction forces is
shown by * (P < 0.05, ANOVA). Values are given as means ± SE. (data modified from III)

4.3 Effect of exercise

4.3.1 Exercise training of mouse (IV)

In order to examine the effect of exercise training on the expression of DHPR, mice were
subjected to treadmill running. The exercise sessions were videotaped and the behaviour
of the animals analysed from the tapes. Each mouse performed a similar kind of training
pattern; however, because of the rather low speed of the treadmill, the running was never
continuous. The sessions also included steady phases, albeit short ones, whenever the
mouse stopped. The treadmill forced the animal to start again, and thus the cycle of
running and standing was still repeated.

After the last training session, the body mass of the mice was measured and the
muscles to be studied were removed and weighed. Interestingly, the body mass of the
sedentary group (41.1 g) was significantly (P = 0.019) lower as compared to the trained group (44.7 g). However, no changes were noticed in the ratios of heart to body and muscle to body mass.

The MHC contents of GAS and RF were analysed from the samples of trained and untrained mice using SDS-PAGE and densitometry. Marked alterations were observed after long-term treadmill running in the MHC composition of GAS. As a whole, the effect of exercise training on muscle fibres was a shift from fast to slow fibres. While an increase is seen in the proportion of fibres which express MHC isoforms IIa and I, the expression of IId and IIB is diminished (IV, Fig. 1, Table I).

After 15 weeks of treadmill running, the relative amount of DHPR protein in GAS increased significantly (P = 0.023) by 21.5% as compared with the non-exercised group. The results are based on immunoblot and densitometrical analysis. No changes in the expression of DHPR were observed in RF and cardiac muscle (IV, Fig. 3). In GAS, the increase in % MHC IIa correlates significantly with the rise in the density of DHPR (P < 0.05).

The qualitative analysis, performed using the fluorophore-conjugated dihydropyridine blocker reveals a higher intensity of fluorescence in the stained cross sections of trained mice as compared to the samples from untrained ones (IV, Fig. 2).

To obtain an estimate of the extent of the expression of Cacna1S gene in mouse skeletal muscle, the tissue samples were subjected to quantitative RT-PCR analysis. In GAS the Cacna1S mRNA level was 66.8% higher (P = 0.024) in the training group as compared to the non-exercised group. No significant changes were observed in RF.

**4.3.2 Downstream migration of farmed salmon (V)**

To get at the physiological role of DHPR in vertebrate taxa other than mammals, the effect of increased muscle activity on DHPR expression was also examined in fish. The densities of dihydropyridine receptors (DHPRs) were accordingly analysed in Atlantic salmon before and after downstream migration in order to see whether intense swimming is associated with variations in the components of the excitation-contraction coupling machinery. The migration, 46.5 km in all, in the river Simojoki from the Tainikoski rapids to the Suukoski rapids took five days.

The swimming speed of fish and the discharge rates of water were measured. Based on these values, the maximal workload of the salmon in the aquarium and in the river was estimated. The results indicate that the relative maximum instantaneous workload of the fish is nearly 1 700 times higher in the river as compared to that in the rotation current aquarium of the fish farm.

The results of Western blot analysis demonstrated that the red muscle tissue samples taken from smolts before release contain two fast isoforms of MHC (V, Fig. 1) with molecular masses of 192 kDa and 187 kDa. After migration, only one isoform (192 kDa) is expressed. There are also changes in the oxidative activity of the red muscle tissue as determined from staining intensity of succinic dehydrogenase (V, Fig. 2). The difference in the SDH activity between the samples taken before and after the migration is statistically significant (P = 0.008). In white skeletal muscle, no staining was observed.
The density of dihydropyridine receptors in cross sections of red muscle, assessed on the basis of the fluorescence intensity, is higher in the migrating as compared to the non-migrating smolts (V, Fig. 3). In these samples, the T-tubules inside the fibres are clearly detectable. Though not so obvious, a similar change in the density of DHPRs is also seen in white muscle.
5 Discussion

Considerable progress has been made during the last three decades in the study of signal transmission process in the skeletal muscle. The primary structure of two fundamental proteins involved, i.e., that of the dihydropyridine and the ryanodine receptor, has been deduced by cloning and sequencing their cDNAs. Advances in membrane physiology have enabled detailed studies of functional properties, pharmacological characteristics and electrophysiological features of the ion channels. In this study, a new methodological approach together with different test arrangements was used in order to elucidate more aspects of dihydropyridine receptor characteristics.

In order to localize and visualize the DHPRs of skeletal muscle, a method using DHP molecules as a probe was developed. This new method is based on the study of Knaus and co-workers (1992) in which DHPs are used to label the DHP receptor domain of the L-type calcium channel. The dihydropyridine probe used in this study was a high-affinity (-)-enantiomer labelled with orange fluorophore that binds selectively to the DHPRs of muscle tissue. Since no specific fluorescence was seen under control conditions, the method proved to be useful in the study of DHPRs.

5.1 DHPRs in developing muscle

The maturation of a striated muscle is a chain of events in which several different components finally form a highly complex contractile structure. The calcium-releasing mechanism essential to the contraction of a muscle fibre, as well as the structures connected to this mechanism, develop during both the pre- and the postnatal stages. It is well established that the most important components involved in this mechanism appear only after the expression of certain structural proteins. Various studies have been undertaken on the development and timing of the appearance of the DHPR channels in the T-tubules of a striated muscle. The results are however partly contradictory, depending on whether the research has been carried out in culture or in the living organism.

Completion of the basic structural framework of the muscle fibre, a prerequisite for the initiation of DHPR expression, was visualized with light and electron microscopy.
Also, the postnatal expression of sodium channels and the relationship between channel expression and conduction velocity of plasma membrane were studied, just to outline a few of the developmental steps which precede the signal transmission in the triad.

According to the results from microscopic analysis, the muscle tissue starts to condense into compact fibre bundles right after birth. During ontogeny, junctions start to form between fibres. According to Perry and Rudnick (2000), the junctions are essential for the differentiation of a muscle fibre. As the tissue becomes more dense, the proportion of extracellular matrix diminishes. Yet this extracellular matrix, composed largely of proteoglycans, has proved important for the muscle tissue differentiation during morphogenesis. After maturation, the function of the matrix is not that essential and the amount of intercellular components is reduced (Velleman 1999).

In addition to the arrangement of muscle filaments to structural units, the membranous compartments also start to organize after birth. In the samples from 15-day-old mice, rows of membrane vesicles are formed between the fibrils. After 20 postnatal days, regular groups composed of three vesicles begin to form, indicating the orderly arrangement of triads. In the samples from adult mice (63-day-old) the triad structures are organized.

The assembly and organization of the adult network of triads is well controlled and follows a certain pattern. During early embryonal development, T-tubules are not yet present. The SR development first takes place independently, while at a later stage, the development of T-tubules and SR is coordinated. On the other hand, junctional SR is assembled simultaneously with the main contractile components (Yuan et al. 1991).

The findings of this study are in keeping with the studies that have described the final stage of maturation of the two membrane systems. The development of membranous compartments involves rearrangement of membrane structures with the result that the triads will be located at A-I junction in mammalian muscles (Gauthier & Hoobs 1986). By the time the triads acquire their final position, the T-tubules become transversely arranged and the SR acquires its adult, longitudinal shape. That the final orderly arrangement is not reached until about 2-3 weeks after birth in mouse (Luff & Atwood 1971) is well documented in this study as well.

The sodium channel plays an essential role in the generation of action potentials sensed by DHPR. In order to monitor the postnatal development of sodium channels, expression of the channels was studied using protein and immunoblot analysis as well as immunohistochemical methods. The results indicate that the density of sodium channels starts to increase after 8 postnatal days (II, Figs. 1, 2). Immunohistochemical studies with rats have shown that the appearance of sodium channels occurs at the age of 17 days, whereas loose-patch voltage-clamp studies revealed the channels at the age of 14 days (Lupa et al. 1993).

Surprisingly, a decrease in the number of sodium channels was observed 9 days after birth. The abundance increased again, however, after postnatal days 15, 20 and 25. Interestingly, the conduction velocity of the muscle followed a similar pattern: though an overall increase in the conduction velocity was observed during the first three postnatal weeks, a clear delay was seen after 9 days postnatally. Nevertheless, these findings show that in the mouse skeletal muscle plasma membrane, the sodium channel is already expressed at birth, and that the number as well as the density of the channels increases markedly during postnatal development. As judged by the conduction velocity
measurements, the electrical properties of the cell membrane are also improved during muscle maturation.

The estimation of the number of DHPRs in the developing muscle was based on the subjective assessment of the intensity of light emitted by the fluorescent tracer that binds to the dihydropyridine receptor (I). Obviously, one possible source of error in the present study comes from the use of a qualitative approach. The results indicate, however, that the number of dihydropyridine receptors increases during postnatal development. In the muscle samples of a newborn mouse, fluorescence was not detected (I, Fig. 1a). This suggests that the number of DHPRs is very low right after birth. In the samples from young mice, on the other hand, the DHPRs were clearly detectable (I, Fig. 1b). In a striated muscle of an adult mouse, DHPRs occur in large numbers (I, Fig. 1c).

It is thus obvious that the number of DHPRs increases considerably during postnatal development. Studies undertaken at the level of protein synthesis and at the molecular level support this argument. Bulteau and co-authors (1997) stated that the expression of mRNA coding the L-type calcium channels of a striated muscle starts immediately after the fusion of myoblasts. According to Kyselovic and co-workers (1994), the expression of mRNA is very weak at the prenatal stage in rat striated muscle and clearly detectable only ten days after birth. The study also showed that the amount of dihydropyridine receptors increases in the intact muscle fibres after birth, and even in the cell culture as a function of time.

The development of the sarcoplasmic reticulum and the expression RyR also have an effect on the number of DHPRs. As previously mentioned, DHP receptors are connected to the RyRs located in the sarcoplasmic reticulum. The connection formed is responsible for converting the electric impulses into mechanical movement, for the release of calcium into cytosol and, furthermore, for the contraction of a muscle. The expression of RyR attains maximal levels after that of DHPR, requiring about 3-4 weeks to reach the high levels that are maintained throughout adult life (Kyselovic et al. 1994).

In a study on the development of muscle cell membranes and junctions, Franzini-Armstrong (1991) demonstrated that, even though the growth of T-tubules as a whole is a slow process, the surface area that forms junctions with the sarcoplasmic reticulum grows rapidly. Hence, despite the slow development of T-tubules, the increase in the number of dihydropyridines can be rapid.

Taken together, the results indicate that prior to the expression of DHPR, the general preconditions for E-C coupling, such as membrane structures and receptors for generation of action potential, are formed. In a patch-clamp study of Gonoi (1993), the relative changes in the densities of voltage-sensitive channels in cultured muscle cells during embryogenesis were measured. The results demonstrate that the expression of both the L-type calcium channel and the adult type sodium channel (Na⁺ toxin-sensitive channel) increase after birth. On the other hand, the expression of embryonal sodium channel (Na⁺ toxin-resistant) diminishes along with the calcium current through T-tubule membrane via L-type calcium channels (Ca²⁺ transient). As can be seen from the foregoing, the results of this study are in keeping with earlier findings and suggest that the density of adult type sodium channels as well as DHPRs increases after birth.
5.2 Location of DHPRs

A light microscopic study of labelled cryosections of muscle samples reveals that DHPRs are located at the periphery of the fibre. In some samples, especially longitudinally cut ones, fluorescence is also detectable inside the cell (I, Fig. 2). In samples from salmon muscles, fluorescence inside the cell is obvious (V, Fig. 3). Previous studies indicate that T-tubules of the skeletal muscle fibre are highly enriched in DHPRs (Fosset et al. 1983, Striessnig et al. 1986, Jorgensen et al. 1989). According to Seigneurin-Venin and co-authors (1996), in a developing muscle the channels are also located in the T-tubules.

Yuan and co-workers (1991) compared the temporal relation between the development of the T-tubular system and the appearance of the $\alpha_1$ subunit ($\alpha_1$-DHPR) of a calcium channel. According to their results, this subunit is expressed in cytosol before the formation of T-tubules, and adopts first a peripheral location in the fibre. These results thus suggest that $\alpha_1$-DHPR membrane molecules are found in peripheral membrane structures prior to their shift to the T-tubules. Along with the development, the vesicles fuse into membrane structures of the T-tubules and the dihydropyridine receptors become membrane proteins of the T-tubular system.

The inward calcium current through the $\alpha_1$-subunit seems to play an important role in muscle development. According to Beam and co-authors (1989) the function of the slow calcium current could be in replenishing the calcium ion stores inside the fibre. It has also been hypothesized that the ion flow is especially important in tetanus (Reynolds & Snyder 1988). Location of the DHPRs near the peripheral areas of the fibre in maturing muscle would thus be optimal in ion transport from the extracellular space.

Posterino and co-workers (2000) have shown that action potentials are capable of travelling longitudinally inside intact muscle fibres. Internal propagation of the impulse would ensure that each sarcomere is synchronously activated, thus producing efficient and powerful contraction of the muscle. These results (1) demonstrate that such an internal longitudinal action potential renders possible a much faster propagation of electrical impulse along the plasma membrane, and (2) point to existence of a longitudinal tubular system (LTS).

Based on microscopic analysis of this work and relevant research data from elsewhere, it appears justified to assume that the DHPRs detected in this study are likely to be located in the membranes of the T-tubules or in the vesicles fusing into the T-tubules near the peripheral area of a fibre (I, III, IV, V). Most of the fluorescence is concentrated in the peripheral areas of the cells, supporting the hypothesis of LTS. On the other hand, DHPRs can also be seen in the inner areas of the muscle fibres. The internal location is particularly well seen in the samples from salmon red and white muscles.

The fish muscle structure differs from that of mammals not only in its gross anatomy, but also in fibre type distribution, fibre size as well as the amount and composition of the connective tissue. Furthermore, the ultrastructure of a fish myofibril differs from that in mammals in having an extensive array of triads at the level of Z-line (Nag 1972, Johnston 1980). In most mammalian and avian muscles, the triads are located at the border of the I and A bands (Flucher 1992). Different distribution of DHPRs in the mouse and the salmon muscle may result from the differences in the location of T-tubules in the myofibril.
In samples extracted from 28-day-old mice, accumulations of strong fluorescence at the border of two cells were detected in addition to even fluorescence (I, Fig. 1b). These spots in all likelihood represent the pericytes – cells which ensheathe the capillaries. In a separate set of experiments (data not shown), a network of capillaries around the muscle fibres was demonstrated with PAS (periodic acid-Schiff) staining. This method stains the basement membrane adjacent to endothelial cells, and here the PAS-positive (i.e., stained) sites corresponded well to those of intense fluorescence.

According to Sakagami and co-workers (1999), exposure of capillaries to serum-derived molecules activates a voltage-dependent pathway for the influx of calcium into the pericytes. Since this pathway employs DHPRs located in the pericyte plasma membranes, it is obvious that the spots of strong fluorescence represent the dihydropyridine accumulations at the borderlines between the cells.

5.3 Distribution of DHPRs

Although the DHPR has been identified in many tissues, descriptions of the intercellular distribution of this protein are few in number. In this study, the organization of DHPR in mouse skeletal muscle tissue stained histologically is reported for the first time. The light microscopic study revealed that distribution of fluorescence is not homogenous in cross-cut muscle samples of mouse (I; IV; thesis, Fig. 8). The result suggests that the density of DHPR varies between different muscle fibres.

Statistical test of correlation between the fibre types and their cross sectional areas indicated that the fast oxidative glycolytic (FOG) fibre type contains the largest number of DHPRs when compared with the slow oxidative (SO) and the fast glycolytic (FG) fibre types (I). In order to correlate the expression of DHPRs more accurately with specific muscle fibre types, separation of muscle fibres was performed according to Hämäläinen and Pette (1993). The results indicate that fibres termed IIA, correspond to the fibres strongly expressing DHPRs (III, Fig. 2).

These results, although more specific, are supported by some previous immunofluorescence labelling studies showing that the intensity of labelling of the α1-subunit is higher in type II (fast) than in type I (slow) fibres (Jorgensen et al. 1989). The findings of this work on DHPR distribution could be partly explained by the differences in T-tubule properties between the two major fibre types. First, the fractional volume of the T-tubular system in the rat intact fibres, for example, is significantly higher in fast fibres as compared to the slow ones (Launikonis & Stephenson 2002). Second, the proportion of junctional T-tubules is higher in fast-contracting than in slow fibres (Eastwood et al. 1982) indicating more dense DHPR appearance in fast fibres. The fibre type specific arrangements of the T-tubule system have also been matched to contractile profiles of adult muscle cells (Takekura & Yoshioka 1993).

Recent studies have provided evidence that E-C coupling characteristics and fibre types based on MHC isoforms are closely interrelated. It has been shown (Goodman et al. 2003) that mechanically skinned pure fast-twitch IIB fibres produce larger T-system depolarisation-induced responses than do pure slow-twitch type I fibres, implying interrelation between optimum force production and MHC isoform type of a single
muscle fibre. This study also demonstrated for the first time that the amount of Ca\(^{2+}\) released from the SR, the rate of Ca\(^{2+}\) release/uptake, and the sensitivity of contractile apparatus to Ca\(^{2+}\) are correlated with the MHC based type of muscle fibre.

The multinuclear nature of a muscle fibre results in regional variation in the gene expression within a fibre. Therefore, distinct subsets of proteins are produced within different parts of the fibres, leading to formation of hybrid fibres (Rosser & Bandman 2003). Since single muscle fibres represent more or less heterogeneous structures, it is no wonder that variation in specific distribution of DHPRs can be found also at the muscle level. The results from fluorescence labelling (I) indicated that the number of DHPRs is different in different muscles. In a sample extracted from whole GAS of mouse, a smaller number of DHPRs was found than in a corresponding sample extracted from the thigh muscle (RF). Furthermore, in the study on the relationship of muscle fibre type composition and contractile responses to DHPR blocker (III), significant differences between the muscles studied were found.

It is well known that the muscle fibre type composition varies from species to species and from muscle to muscle. Also, the activity and the function of a muscle have an effect on its fibre type composition. Muscles with a high proportion of type I fibres are usually involved in the maintenance of posture, whereas muscles with a high type II content are used for movements requiring high power output (Goldspink 1977). Generally, the skeletal muscle tissue of a vertebrate is an extremely heterogeneous tissue composed of a variety of fibres. The muscles studied in this thesis, i.e., GAS, RF and GLU, differ from each other not only in size but also in relative power output and function. This difference can be detected in fibre type composition and, furthermore, in the number of DHPRs.

According to the results from histochemical and immunoblot analysis as well as previous studies (Ariano et al. 1973, Sugiura & Murakami 1990, Hämäläinen & Pette 1993), the GAS has a high type IIB content, whereas both RF and GLU are predominantly composed of types IIA and IID. The contraction response studies showed that the DHPR blocker nifedipine inhibiting effect was significantly higher in RF and GLU than that in GAS. These data reveal that the same amount of blocker causes different response in muscles with different fibre type composition. The GAS with low IIA content has a weak response to the added channel blocker, whereas on the muscles with high type IIA content the effect of the blocker is much stronger, i.e., the contraction force is effectively reduced. The findings are deducible from the fibre type specific character of DHPR, also resulting in a muscle type specific feature of the DHPR distribution.

These data are consistent with the earlier electrophysiological studies where the vaseline-gap technique was used in order to record slow calcium currents in fast- and slow-twitch muscles. It could be inferred on the basis of the results that the slow calcium current through the T-tubule membrane is much smaller in slow fibres than in fast fibres. Furthermore, the experiments on the binding of labelled dihydropyridine (+)PN200/110 indicated that fast-twitch muscles had about 2 to 3 times more specific DHP binding sites than did the slow-twitch muscle (Lamb & Walsh 1987).

The nifedipine binding on DHPR and, furthermore the effect on muscle contraction was clearly detectable (III) despite the fact that based on previous studies, blocking the inward calcium flow has no effect on muscle contraction. Nifedipine is an allosteric modulator binding to the inactive state of the channel molecule. The modulation of
channel activity is quite complex depending primarily on drug structure, membrane potential, frequency of stimulation, and the resulting channel conformational states (Hockerman et al. 1997). Previous studies have shown that at least D-cis-diltiazem, a DHP derivative, changes the channel architecture (Glossmann et al. 1984). Based on this and the present data, nifedipine could also induce a change in the conformation of DHPR thus preventing the voltage-controlled release of Ca$^{2+}$ from SR.

In contrast to mammals, the musculature of fish shows three discrete regions, which are composed of functionally different fibre types. The bulk of the myotome consists of white muscle, historically classified according to colour. The white muscles are composed of fast oxidative glycolytic or fast glycolytic fibres with intermediate or low amounts of myoglobin. In fish with a round body, red oxidative fibres with high concentrations of myoglobin form a superficial sheet of muscle adjacent to the lateral line. Between the white and red muscle layers, a third fibre type, termed pink, is expressed (Johnston 1980). Based on the organization of the musculature in fish, a homogenous distribution of DHPRs in cross sections from salmon muscles was anticipated. This was also the case as seen in V, Fig. 3. However, the densities of DHPRs in the white and red muscle samples from farmed smolts were quite similar. The differences were small and hardly significant.

5.4 Effect of increased muscle activity on DHPR content

The determination of DHPR expression and distribution gives an overall indication about the connection of this protein to the general properties of a muscle fibre. Besides having an influence on the performance of a single fibre, the content of DHPRs in a whole muscle contributes to the muscle function. Intensive research into the effects of various conditions, such as innervation, aging and hormone treatment, has clearly established the exceptional adaptive potential of muscle tissue. In order to examine the plasticity of DHPR expression, variations in the density of the molecule were studied during altered neuromuscular activity.

5.4.1 Exercise training of mouse (IV)

In order to study the effect of endurance training on the expression of DHPR in skeletal muscle of a mammal, muscle samples were taken from mice subjected to a 15-week aerobic exercise program. Additionally, the changes in the myosin heavy chain content were examined so as to be able to correlate the changes between MHC isoforms and DHPR expression. The results showed a significant increase in the DHPR expression in GAS after training. Furthermore, the training induced changes in MHC protein content in favour of MHC IIa. The increase correlated significantly with the one in the amount of DHPR. In RF no changes were observed.

In mammals, the contractile properties of the muscle can be altered in two ways during increased muscular activity. These include the transition of fibre phenotype and selective hypertrophy of a given fibre type. As a result, the efficiency and economy of energy
usage of a muscle can be adapted during exercise (Goldspink 1998). As previously indicated, the expression of different MHC genes has been shown to be tightly regulated by several factors, such as electrical stimulation (Pette & Vrbova 1986), stretch and overload (Goldspink et al. 1992). In mice, the modification to increased muscle load is related to a shift in MHC composition towards the slower fibre types (Zhan et al. 1999, Allen et al. 2001), which was also found in the present study in GAS. On the other hand, according to the results from the image analysis, the training also resulted in the formation of hybrid fibres, i.e., not pure fibres alone.

The fast oxidative glycolytic fibres, such as type IIA, have at least a moderate contraction speed and they endure long periods of exertion without fatigue. The fibres have several mitochondria, which render the cell type enduring (Goldspink 1977). Moreover, the oxidative capacity is superior to the fast glycolytic type of fibre as measured with oxidative activities (Close 1972, Roneus et al. 1992, Mattson et al. 2002). Because of these fibre type characteristic properties, type IIA is fairly fatigue-resistant and recovers rapidly after exertion. Indeed, the central role of fibre type in contraction performance has been broadly documented (Galler et al. 1994, Bottinelli et al. 1996, Geiger et al. 2000, Weiss et al. 2001). In keeping with this, it seems reasonable that if the muscle is to perform rapid yet prolonged contractions, the relative amount of type IIA in muscles with increased activity is augmented. This is also documented in recent studies with mouse (Allen et al. 2001) and human subjects (Putman et al. 2004).

The amount of the metabolic energy expended and the related mechanical work performed during locomotion is fundamental for assessing muscle economy and the optimization of the movement. In order to adjust their efficiency to required forces, velocities, and endurances, muscles are composed of a mixed population of fibres with different functional capabilities. Physically active muscles have a predominance of fast fibres, whereas tonic muscles used in maintaining posture mainly have slow fibres with a high oxidative capacity. Muscles that consist primarily of fast oxidative glycolytic fibres have adjusted to frequent, rapid movements (Goldspink 1977).

The effect of endurance training on the muscles studied in this thesis depends, to a large extent, on the inherent characteristics of the muscles themselves. Previous studies on rats indicate that both leg muscles, RF and GAS, are intensively activated during running (Laughlin & Armstrong 1982). However, a major difference between these two muscle types is a markedly higher proportion of type IIA fibres in RF (Ariano et al. 1973).

As mentioned earlier, type IIA fibres have the most adequate endurance properties of the known fast-twitch fibres. Therefore, the initial properties of RF are better suited for prolonged training than those of GAS. The GAS, along with the soleus and plantaris muscles, produces an extension moment at the ankle. The proportion of IIB fibres in GAS is high, indicating a central role in force production during high intensity muscular activity. Indeed, an increase in the speed of locomotion appears to be associated with an increase in the power, velocity, and work done by GAS, as indicated by studies in cats (Prilutsky et al. 1996).

Regarding the adaptive potential of muscles, it seems obvious that the muscles better suited to mechanical work under anaerobic conditions producing fast movements undergo the most distinct improvements in endurance properties as a result of long-term aerobic
training. This was also the case in the present study, in which the training had the most significant effects on the GAS, whereas in RF no significant changes were observed.

Calcium ions are the primary mediators of skeletal muscle contraction. Given the central role of calcium in skeletal muscle performance, the effect of endurance training on the components of \( \text{Ca}^{2+} \) release system is not that well studied. There are, however, several observations on significant increases in GAS SR protein \( \text{Ca}^{2+} \)-ATPase (SERCA) mRNA expression after training, indicating an effective \( \text{Ca}^{2+} \)-reuptake into the SR. According to the results, the increase is related to the 2a isoform of SERCA protein expressed in slow-twitch muscle referring to the shift from fast-to-slow fibres (Kubo et al. 2003). In other major components, such as ryanodine receptor or calsequestrin, no published data about the impact of endurance exercise on the expression of these proteins were found. On the other hand, it has been shown that intermittent sprint training associated with fatigue enhances SR \( \text{Ca}^{2+} \) release via enhanced SR content and increased density of RyR (Ørtenblad et al. 2000).

In the current study, an increase was observed in the density of DHPR after intermediate endurance training. The results are supported by previous studies with rat, in which regulation of DHPR in protein level by exercise training was noted (Saborido et al. 1995). The interesting finding in the present study was the significant correlation between the increment in both DHPR expression in mRNA as well as protein level and fibre type IIA portion in GAS after training. The result complements the previous findings by indicating the fibre type IIA specific character of DHPR.

5.4.2 Migration of salmon (V)

In order to move to the left in the phylogeny, fish was also chosen for the study on the effect of endurance training. In addition, the changes taking place in the natural environment of the animal were also investigated.

The fish muscles are specialised not only in different modes of swimming, but also in demands of the physical factors of the environment. In salmonids, also the stage of development has an effect on the mechanistic and molecular properties of the swimming performance (Coughlin et al. 2001). In order to study the exercise adaptation after smoltification on muscular level, the relative number and distribution of myosin heavy chain isoforms as well as the changes in the DHPR density were analysed before and after the downstream migration of Atlantic salmon. Furthermore, an ecophysiological approach was taken to clarify the migration behaviour of smolts.

To find out the level of active movement of fish in the river compared to that in the culture, the maximal workload was estimated on the basis of swimming velocity in the aquarium and in the river. According to the results, the travel time of salmon migrating under natural conditions in the river Simojoki was longer than would be that of an object floating freely in the water current, i.e., 5 days vs. 26 hours, respectively. This, along with the previous studies (Thorpe & Morgan 1978, Hansen & Jonsson 1985, Bourgeois & O’Connell 1988, Moser et al. 1991, Greenstreet 1992, Fängstam 1993, Lacroix & McCurdy 1996) indicates that fish are, at least part of the time, swimming actively against the current. The calculated relative difference in workload between the released
and farmed smolts, although only indicatory, leads to the assumption of increased muscle activity of migrating smolts.

As a result of the migration, several changes were observed on molecular level of the swimming muscles. The most apparent alteration was the transition of the muscle fibre phenotypic profile. According to Karasinski (1993), the MHC expression in fish skeletal muscles is known to be very varying and dependent on fibre type, developmental stage and fish species. In addition, fibres of fish skeletal muscle are also dynamic structures, similar to those in mammalian muscles, with an exceptional ability to accommodate different situations, for example increased muscular activity (Coughlin et al. 2001). In the present study, a decrease in the relative number of MHC isoforms from two to one after migration is reported. There exists similarity of MHC sequence homology between fish and mammalian muscle (Lutz et al. 1998, Weaver et al. 2001). Based on this, the results indicate that the transition trend in fish muscle is also from fast to slower isoforms due to exercise, as in mammalian muscle (Pette & Staron 2001).

The changes in MHC isoform content were associated with DHPR density, as revealed with the histological analysis. The results indicated that there is an obvious increase in the density of DHPRs in salmon muscle after downstream migration. Moreover, the increase seems to take place mainly in the internal parts of the fibres. As described before, the level of the DHPR density in white and red muscles of farmed smolts was somewhat similar before the migration. However, after the release into the river and the five days’ seaward migration, the DHPR density of red muscle in particular was increased, although a clear augmentation was also seen in the amount of DHPRs in the white muscle as well (V, Fig. 3).

The red muscles of salmon are used in steady swimming, whereas the white muscles produce short-term bursts (Bond 1979). Interestingly, the observation suggests that on the contrary to the mammalian muscles, the DHPR density after increased activity of muscles is more pronounced in slow oxidative fibres as compared to the fast glycolytic ones.

Although the sequence of DHPR is very similar to the mammalian counterpart (Grabner et al. 1991), the different subunit composition and the size of the receptor may have an effect on the function of the protein, thus having a different kind of role in the skeletal muscle of fish as compared to the muscle of mammals. Furthermore, the special feature of fibre arrangement providing the fish with three different swimming speeds gives rise to some fundamental differences between the use of muscles in fish and mammals. Although the white fibres account for 70 to 80% of the swimming musculature in salmon, all the muscle is inactive most of the time, while the red muscles along with the pink fibres are more efficiently recruited (Johnston 1980). This difference in the muscle activity between white and red muscles should have an impact on the density of DHPRs after swimming in natural environment.
6 Conclusions and further study visions

This work consists of five studies designed to characterize certain features of dihydropyridine receptors in the vertebrate skeletal muscle.

The results show that during postnatal development the level of DHPR expression is gradually increased in the mouse skeletal muscle. By the time of DHPR appearance, the expression of sodium channels has started and the basic structural components of the muscle fibre are clearly detectable.

Furthermore, the present data provide evidence for the fact that the expression of DHPR is not uniform in different fibre types but selectively expressed in type IIA fibres. In other words, fast fibres with high glycolytic capacity and high levels of oxidative enzymes also have a high density of L-type calcium channels. In addition to the fibre type specificity, the uneven distribution of DHPRs is also seen at the muscle level. Since type IIA fibres are rather fatigue resistant, the high level of DHPRs is, presumably, one of the necessary conditions for rapid, strong and continuous muscle contraction.

The fibre type specificity of DHPR is also reflected as changes in the muscle tissue after endurance training. Significant increases in the DHPR mRNA level as well as protein level were observed in the gastrocnemius muscle after moderate endurance training. The results suggest that DHPR expression is upregulated as a result of increased muscular activity. Moreover, the changes in the expression correlated well with the increase in the IIA fibre type content, thus confirming the relationship between DHPR and type IIA fibre.

The adaptive response of the excitation-contraction coupling machinery to increased muscular activity in fish swimming muscles was also studied. Different test arrangements were set up in order to observe the effects of exercise in natural environments, and to clarify the downstream migration behaviour of salmon. According to the results, the hatchery release is manifested as adaptive structural changes in swimming muscles of the salmon. Migration induced a transition of fibre phenotypic profile from fast to slow and enhanced the expression of DHPRs. On the basis of the observed changes at the myofibrillar protein level, it was concluded that the seaward migration of smolts is not just passive drifting, but requires considerable muscle work. The localization and distribution of DHPR is similar to that in mammalian muscle. Contrary to the mammalian muscles, however, after active use of muscles the DHPR density is more pronounced in
slow oxidative fibres as compared to the glycolytic ones. This, along with the results on mice, indicates a strong correlation between the level of muscle activity and the density of DHPR.

Skeletal muscle is among the best-characterized animal tissues. However, further research is required to fully understand the facility with which a muscle performs movements and responds to a variety of stimuli. Since all muscle fibres use calcium ions as their main regulatory and signalling molecule, gaining structural and functional insights into the Ca\(^{2+}\) regulating apparatus has become essential. Therefore, further studies on the expression and adaptive changes of proteins besides DHPR involved in Ca\(^{2+}\) signalling and handling, such as RyR and SERCA, would be important. Furthermore, complementary studies on the evolution of the main elements of the calcium cycle machinery are needed in order to determine whether the components have conserved or species specific functions.

As I see it, it is not difficult to underline the importance of further studies on the tissue that basically makes life go.
References


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64


