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REGULATION OF STRIATED MUSCLE CONTRACTION

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To my family with love

ABSTRACT

Background and Aims: Muscle contraction involves cross-bridge interaction between actin and myosin filaments, which is regulated by variations of intracellular $[Ca^{2+}]$ and influenced by several external control systems. To characterize the mechanism of contraction, we have studied the effects of pharmacological compounds influencing the actin-myosin interaction and/or Ca^{2+} activation systems. We examined the effects of blebbistatin, a novel inhibitor of the actomyosin ATPase, in the organized contractile system of muscle (papers I and II). Cardiac contraction can be influenced by extracellular factors and in paper III, we examined the inotropic action of UTP and UDP, which can be released under ischemic conditions. The recent developments in molecular biology and the genetic characterization of several muscle diseases have created a need to link specific genes and proteins to functions in muscle. The zebrafish (*Danio rerio*) has become a useful vertebrate model organism in this context. The main aim of paper IV was to develop techniques for studies of skeletal muscle in the zebrafish larvae and to characterize its mechanical and structural properties. **Results and Conclusions:** In paper I, blebbistatin inhibited contraction of the cardiac preparations of the mouse with the half-maximal inhibitory concentration in the micromolar range. Using permeabilized cardiac preparations, we showed that blebbistatin did not influence Ca^{2+} -sensitivity of the contractile filaments. Using patch clamp technique, we showed that blebbistatin neither interfered with action potential duration nor Ca^{2+} influx. Thus, in mouse heart, blebbistatin directly inhibits actin myosin interactions. In Paper II, we extended the observation of blebbistatin's ability to bind differently to several isolated myosin isoforms *in vitro*: the contractions in a range of skeletal, cardiac and smooth muscle tissues, all showing different myosin isoform profiles, were differently inhibited by blebbistatin. Thus, blebbistatin's selectivity for different myosin isoforms is also present in the organized contractile system. Using permeabilized skeletal muscle where active force measurements were combined with small angle x-ray diffraction (synchrotron sourced), we further showed that blebbistatin interacted with a nucleotide-bound myosin state and trapped the myosin cross-bridge in a configuration dissociated from actin. In paper III, we examined extracellular control mechanisms for cardiac contraction focusing on pyrimidines which are released in the human during ischemia. They act on isolated cardiac muscle in a receptor-specific way through an IP_3 -pathway which behaves independently from cAMP. Thus, we established that pyrimidines are an external control mechanism for cardiac shortening acting independently of the usual inotropic mechanisms. In paper IV, we developed a novel method to study structure and mechanical properties of skeletal muscle of zebrafish in the larval stadium. Single twitch and tetanus contractions could be recorded at an optimal sarcomere length of 2.15 μm . Clear equatorial x-ray diffraction patterns could be recorded showing that the myofilaments were mainly arranged in parallel to the long axis of the larval preparation. A smooth tetanus was observed at a high stimulation frequency. Repeated tetanic stimuli uncovered that fatigue developed rapidly in these muscles. These results show that the zebrafish larvae muscle have a fast muscle phenotype. Permeabilized muscle preparations showed a lower Ca^{2+} -sensitivity of contraction compared with mouse skeletal muscle. Thus, zebrafish larval skeletal muscle displays a fast contractile profile where a high cytoplasmic Ca^{2+} -concentration for contraction is combined with rapidly developing fatigue. The study of the influence of different levels of muscle gene expression on the mechanical behavior of the tissue has with these experiments become a distinct possibility.

LIST OF PUBLICATIONS

- I. **Dou Y**, Arlock P, Arner A. Blebbistatin specifically inhibits actin-myosin interaction in mouse cardiac muscle.
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- III. Wihlborg AK, Balogh J, Wang L, Borna C, **Dou Y**, Joshi BV, Lazarowski E, Jacobson KA, Arner A, Erlinge D. Positive inotropic effects by uridine triphosphate (UTP) and uridine diphosphate (UDP) via P2Y2 and P2Y6 receptors on cardiomyocytes and release of UTP in man during myocardial infarction.
Circulation Research 2006, 98: 970-6
- IV. **Dou Y**, Andersson-Lendahl M, Arner A. Structure and function of skeletal muscle in zebrafish larvae.
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LIST OF ABBREVIATIONS

4-AP	4-aminopyridine
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AM	actin-myosin complex
BDM	2,3-butanedione monoxime
BTS	N-benzyl-p-toluene sulphonamide
cAMP	cyclic adenosine monophosphate
EDL	extensor digitorum longus
EGTA	ethylene glycol tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMM	heavy meromyosin
I_{Ca}	Ca^{2+} current
IC_{50}	the half maximal (50%) inhibitory concentration
IHC	immunohistochemistry
IP_3	inositol trisphosphate
MHC	myosin heavy chain
ML	muscle length
PCR	polymerase chain reaction
P_i	inorganic phosphate
PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
PSS	physiological salt solution
SL	sarcomere length
SR	sarcoplasmic reticulum
SERCA	sarcoplasmic reticulum Ca^{2+} ATPase
TEA	tetraethylammonium
Tm	tropomyosin
Tn	troponin
UDP	uridine diphosphate
UTP	uridine triphosphate
V_{max}	maximal (unloaded) shortening velocity

1 INTRODUCTION

The striated muscle cells of the heart and skeletal muscles contain ordered overlapping arrays of thick and thin filaments. The thick filaments are composed of myosin molecules with helically arranged heads projecting outwards from the filament. The thin filaments, which are anchored to the Z discs, contain actin and the regulatory proteins, tropomyosin and troponin. The interaction between myosin and actin is the molecular basis of contraction. Force and shortening of the muscle occur as a result of a number of reactions involving an elevation of the intracellular $[Ca^{2+}]$, Ca^{2+} binding to regulatory proteins, structural changes of myofilaments and the actin-myosin interaction (Cooke 1997).

1.1 THE CROSS-BRIDGE CYCLE

The cross-bridge cycle, i.e. the cyclic interaction between myosin and actin, forms the basis of muscle contraction. This is a multistep process involving binding and hydrolysis of ATP and the release of the products ADP and inorganic phosphate (P_i). As illustrated schematically in Fig. 1, ATP binding to the myosin head of the actin-myosin complex (AM) causes M-ATP to release from actin. ATP is hydrolysed to ADP and P_i , when the cross-bridge is dissociated generating the M-ADP- P_i complex. M-ADP- P_i can interact weakly with actin (forming A-M-ADP- P_i). The “power stroke” which is the structural change in the cross-bridge that generates force and shortening, is thought to occur when P_i is released from the myosin head. The resulting complex (AM-ADP $_i$) undergoes an isomerisation (AM-ADP $_{ii}$) (Smith, Geeves et al. 2008). Then, ADP is dissociated from the myosin head followed by ATP binding and another cycle. Whereas force is coupled to release of P_i (Takagi, Shuman et al. 2004) and the shortening velocity is rate limited by the off-rate of the ADP release (Weiss, Rossi et al. 2001).

The motor domain of myosin which includes the actin- and ATP-binding sites forms the cross-bridge and generates the molecular motion during its interaction with actin. It is believed that the different domains move during the contraction process correlated with different nucleotide-bound states of the myosin head (Geeves and Holmes 1999). X-ray crystallography has suggested that the switch 2, one of the ATP-binding site contacts on the myosin head, exists in two conformations: “closed” and “open”. The “open” conformation of switch 2 is observed when ATP or ADP is bound to the nucleotide site. The ATP hydrolysis reaction takes place in the “closed” conformation (Geeves and Holmes 1999). The transition of the cross-bridge to the strongly binding force-generating states is accompanied with conformational changes from “closed” to “open” (Fig. 1). Furthermore, the “closed” conformation was found to be associated with ordered helical array of cross-bridge projections on the thick filaments (Xu, Gu et al. 1999). The movement of the cleft between the upper and lower 50kDa myosin domains is coupled to actin binding and dissociation. Fluorescence spectroscopic data and high resolution cryo-electron microscopy study suggest that actin binding induces closure of the cleft and the cleft is closed in the strong actin binding state (Conibear, Bagshaw et al. 2003; Holmes, Angert et al. 2003).

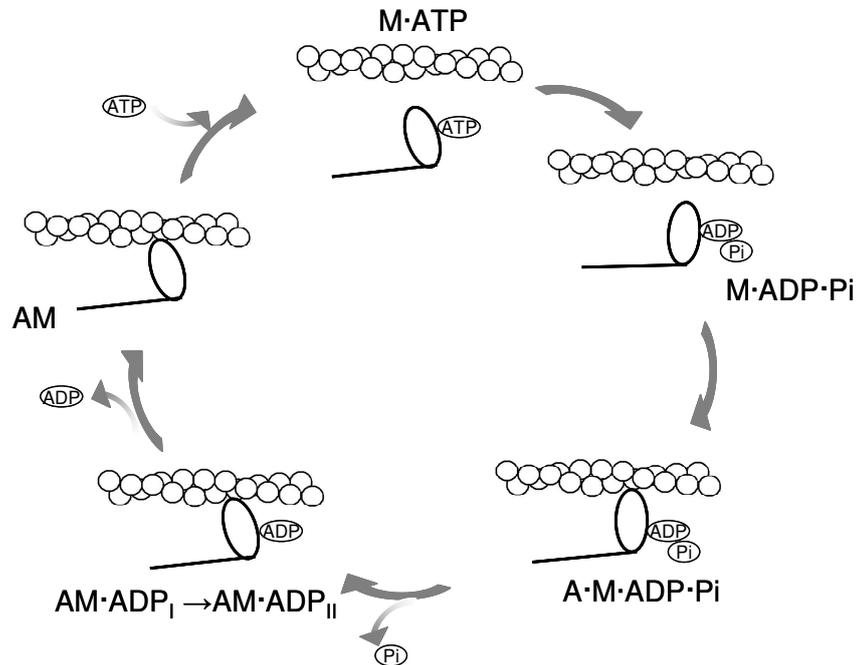


Figure 1. Schematic illustration of the biochemical and structural changes during the cross-bridge cycle when myosin (M) interacts with actin (A) and hydrolyze the substrate ATP to the products ADP and inorganic phosphate (P_i). The scheme was adapted from (Rayment, Holden et al. 1993; Cooke 1997).

1.2 MYOSIN DIVERSITY AND MUSCLE FIBER TYPES

Myosin, the main motor protein of muscle, belongs to a superfamily including a large number conventional and non-conventional myosins, which have several functions in motility and signaling of different cellular systems. The myosins that can form contractile filaments in muscle are denoted “conventional” myosins or Type II myosin (Berg, Powell et al. 2001). Myosin II is composed of two heavy chains and four light chains. The motor domain on the myosin II heavy chain includes the actin- and ATP-binding sites, as discussed above. A neck domain binds the light chain and a tail domain participates in filament formation. The molecular structure of the myosin head has been determined using crystallography (Rayment 1996). Several different myosin heavy chain (MHC) isoforms have been identified. Based on sequence analysis of the motor domains, the cardiac and skeletal myosins are classified into one group whereas smooth and non-muscle myosins fall into another. At least four isoforms of the MHC (MHC-I, MHC-IIa, MHC-IIb, MHC-IIx) are expressed in adult mammalian skeletal muscle and two isoforms (MHC- α and MHC- β) in heart (Lompre, Nadal-Ginard et al. 1984). The cardiac MHC- β is the same gene as the slow skeletal MHC-I. (Lompre, Nadal-Ginard et al. 1984; Schiaffino and Reggiani 1996). The smooth muscle myosin heavy chain is generated by one gene SM-MHC. Three non-muscle myosin II genes NMIIA, NMIIIB and NMIIIC are found (Berg, Powell et al. 2001).

Skeletal muscles have classically been divided into “red” and “white” muscle types, related to different myoglobin levels. Based on histochemical analysis of myofibrillar ATPase, muscle fibers can be distinguished as type I (slow) and type II (fast) fibers. More recently, using electrophoretical, immunological and genetical methods, the myosin heavy chain isoforms discussed in the paragraph above can be identified. The functional properties of muscle fibers, such as maximal (unloaded) shortening velocity and ATPase activity have been correlated with expression of myosin isoforms of different fiber types (Pette and Staron 2000). This correlation has been found in several species including human (Larsson and Moss 1993). The maximal shortening velocity is low in skeletal slow twitch fibers (expressing MHC-I). These fibers are also more resistant to fatigue. In contrast, the fast twitch fibers (expressing MHC-IIb) have a higher maximal shortening velocity and fatigue more easily (Bottinelli and Reggiani 2000; Pette and Staron 2000). The myocardial muscle expresses MHC- α and MHC- β (Schaub, Hefti et al. 1998). The relative expression varies with species. The cardiac ventricular muscle in the slower hearts from large animals, including humans, expresses more MHC- β whereas the cardiac muscle of smaller animals expresses more MHC- α (Schiaffino and Reggiani 1996). Also differences exist within the heart. The pig cardiac atrium, which contains relatively more MHC- α , has faster contractile kinetics than the pig ventricular myocardium, which expresses MHC- β (Morano, Arndt et al. 1988; Svensson, Morano et al. 1997). The contractile kinetics of striated muscle are also modulated by the light chains (Hernandez, Jones et al. 2007). In particular, the expression of the essential light chain appears to be a modulator of cardiac performance (Schaub, Hefti et al. 1998).

Smooth muscle myosin heavy chain (SM-MHC) is the product of one gene (Berg, Powell et al. 2001), but a large variability on contractile kinetics exists between different smooth muscles (Malmqvist and Arner 1991). The molecular mechanisms for this variability are still not clarified. Both expression of different essential light chain isoforms (Malmqvist and Arner 1991; Huang, Fisher et al. 1999) and of a 7-amino acid splice variant of myosin heavy chain (Kelley, Takahashi et al. 1993) might alter the contractile kinetics. Possibly, these isoforms vary in a coordinated manner (Arner, Löfgren et al. 2003). Recently, it was reported that non-muscle myosins (NMIIA and/or NMIIIB) also can form filaments and participate in contraction of smooth muscle (Morano, Chai et al. 2000). The non-muscle myosins appear to have a contractile function in new-born smooth muscle (Morano, Chai et al. 2000; Arner, Löfgren et al. 2003; Löfgren, Ekblad et al. 2003) and might also be functional in some adult smooth muscles (Rhee, Ogut et al. 2006).

1.3 CALCIUM AND REGULATION OF CONTRACTION

Muscle contraction is triggered by an increase of intracellular calcium concentration. The activator Ca^{2+} is recruited from extracellular and intracellular sources (Rüegg 1992). In skeletal muscle, depolarization of the T-tubular membrane leads to a complex structural interaction with a conformational change in the dihydropyridine receptor and an associated activation of the ryanodine receptor in the sarcoplasmic reticulum (SR), which releases Ca^{2+} to the cytoplasm (Bannister 2007). In cardiac muscle, the structure of the dihydropyridine-ryanodine complex differs from that in skeletal muscle (Franzini-Armstrong, Protasi et al. 1998) and Ca^{2+} influx via L-type Ca^{2+} channels induces a Ca^{2+} release from SR (Eisner, Diaz et al. 2004). Contraction is terminated and the striated muscle relaxed when Ca^{2+} is transported back into the SR by the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump. In cardiac muscle, other

mechanisms also extrude Ca^{2+} from the cells. Although variation in the intracellular $[\text{Ca}^{2+}]$ is the main modulator, several other control systems can affect the contraction. In the myocardium, inotropic effects can occur via altered intracellular Ca^{2+} levels and direct effects on the contractile filaments. This involves activation membrane receptors and associated changes in the concentration of second messengers. It is well known that β_1 -adrenergic receptor stimulation has a positive inotropic effect via activation of a GTP binding protein (G_s), which stimulates adenylyl cyclase, increases cAMP and activates protein kinase A (PKA). PKA phosphorylates several proteins in the excitation contraction coupling mechanism such as phospholamban, L-type Ca^{2+} channels, troponin I (Rüegg 1992; Zaugg and Schaub 2004). Several other factors, e.g. nucleotides (ADP, UTP and UDP, as discussed below in Section 1.5), affect myocardial contractility through other pathways, e.g. production of inositol trisphosphate (IP_3) via activation of phospholipase C (PLC) and via protein kinase C (PKC) pathways (Zaugg and Schaub 2004).

The final event in activation of striated muscle contraction is the altered position of tropomyosin (Tm) caused by Ca^{2+} binding to the troponin (Tn) complex. When intracellular $[\text{Ca}^{2+}]$ is elevated, Ca^{2+} binds to troponin C (TnC) inducing a conformational change that allows Tm to move and expose myosin-binding sites on actin. Even if Ca^{2+} binding and structural changes in troponin cause tropomyosin to move and initiate cross-bridge attachment, the cross-bridge attachment also causes tropomyosin to move and thus contribute to activation. This cooperative effects on skeletal muscle thin filaments was originally observed by Bremel and Weber (Bremel and Weber 1972). Thus when myosin heads bind to actin, the alteration of Tm position is transmitted to the neighbouring actin monomers in a cooperative manner (McKillop and Geeves 1993). Ca^{2+} binding to TnC promotes cross-bridge binding, while the cross-bridge binding increases Ca^{2+} affinity to TnC as well (Güth and Potter 1987; Hofmann and Fuchs 1987). This mechanism of cooperative activation may be more important in cardiac muscle than in skeletal muscle (Wang and Fuchs 1994). Although smooth muscle has tropomyosin and potential thin filament regulatory proteins, the main Ca^{2+} regulation of smooth muscle and non-muscle myosin is via the thick filaments and myosin light chain phosphorylation (Horowitz, Menice et al. 1996; Somlyo and Somlyo 2003; Andersson and Arner 2004). In principle, Ca^{2+} activates the myosin light chain kinase that phosphorylates the regulatory myosin light chains and initiates cross-bridge cycling. Relaxation is caused by dephosphorylation by a myosin light chain phosphatase.

1.4 INHIBITION ON ACTIN-MYOSIN INTERACTION

As discussed above in Sections 1.1-1.3, contraction and relaxation of muscle is the result of a complex series of reactions involving the muscle membrane and its receptors, the Ca^{2+} -translocation systems, the thin and thick filament associated regulatory systems and the actin-myosin interaction. As indicated above, a large variability in all of these components exists between the different muscle types: skeletal, cardiac and smooth. Also within each muscle type, differences in the excitation contraction coupling and cross-bridge interaction exist, e.g. between fast and slow twitch striated muscle fibers, between atrial and ventricular cardiac muscle and between different smooth muscle tissues. Several pathophysiological changes are associated with alterations in membrane properties, Ca^{2+} -activation and contractile protein function. One example is hypertrophic cardiomyopathy where changes in

several structural, regulatory and motor proteins have been found (Marian and Roberts 2001; Chang, Parvatiyar et al. 2008). To experimentally characterize these processes in different normal and diseased muscle tissues, specific inhibitors, or activators, of each process in the muscle contraction are needed. Several agonists and inhibitors of membrane receptors, of calcium translocation processes and of enzymes in the activation/deactivation are available and also used clinically. The products, inorganic phosphate and ADP, affect muscle contraction and some compounds like vanadate inhibit force generation (Fuchs and Wang 1991; Jaworowski, Ozturk et al. 1999), but in general, compounds that specifically influence of the actin-myosin interaction in muscle are very sparse. Such compounds are of considerable interest when e.g. membrane events, cell signaling or metabolic processes should be experimentally dissociated from the actin-myosin interaction. Also if compounds with myosin isoform specificity would be available, the relative role of each motor component could be resolved in muscles containing different myosin isoforms. The future clinical use can only be speculated on. In principle, probing the actin-myosin interaction with a specific compound might give new knowledge regarding the functional consequences of genetic alterations in the actin-myosin interface. In addition, inhibition of actin-myosin might be used to alter tissue metabolism or contractile performance in some clinical states.

1.4.1 BDM

BDM (2,3-butanedione monoxime), was originally considered a chemical phosphatase and has been used as a phosphatase activator in cardiac cells (Coulombe, Lefevre et al. 1990). It has negative inotropic effects (Wiggins, Reiser et al. 1980) and it was suggested that it specifically inhibited actin-myosin interaction in cardiac and skeletal muscle in a concentration dependent manner (Horiuti, Higuchi et al. 1988; Alpert, Blanchard et al. 1989; Blanchard, Smith et al. 1990). BDM has been applied in experimental studies on cardiac heat production and oxygen consumption to dissociate contraction and activation dependent energetics (Yaku, Slinker et al. 1993) and in more applied studies of cardiac preservation for heart transplantation and of cardiac ischemia (Stringham, Paulsen et al. 1992; Warnecke, Schulze et al. 2002). Interestingly, BDM appears to have some muscle type selectivity, since smooth muscle is much less affected than skeletal muscle (Österman, Arner et al. 1993). However, BDM depresses force generation in smooth, cardiac and skeletal muscle via several mechanisms, not only by inhibition of actin-myosin interaction and cross-bridge cycling. Several of the activation steps in muscle, including phosphorylation of myosin light chains, Ca^{2+} influx, Ca^{2+} release from SR, Ca^{2+} sensitivity of myofilaments (Gwathmey, Hajjar et al. 1991; Österman, Arner et al. 1993; Siegman, Mooers et al. 1994; Zhao and Kawai 1994; Zimmermann, Boknik et al. 1996) are affected by BDM. Although BDM in some concentration ranges might have specific effects on actin-myosin interaction, alternative sites of action must be considered in studies using this compound.

1.4.2 BTS

BTS (N-benzyl-p-toluene sulphonamide) was identified using screening of a small-molecule library for inhibitors of rabbit muscle myosin II subfragment 1 actin-stimulated ATPase activity (Cheung, Dantzig et al. 2002). The compound was reported to suppress active force of fast twitch skeletal muscle from frog and rabbit, but was much less effective in slow twitch skeletal and cardiac muscle. It does not

interfere with unloaded shortening velocity or the Ca^{2+} transients (Cheung, Dantzig et al. 2002; Pinniger, Bruton et al. 2005). Kinetic studies have shown that BTS decreases actomyosin ATPase rate and inhibits the P_i release and lowers the affinity of M-ADP- P_i and M-ADP for actin (Shaw, Ostap et al. 2003). The specific inhibitory effect of BTS on actin-myosin interaction has been exploited in studies of ATP utilization of the SERCA pump, of contraction related ATP consumption and glucose transport in skeletal muscles (Young, Harwood et al. 2003; Zhang, Andersson et al. 2006; Sandstrom, Zhang et al. 2007). However, since BTS appears to be comparatively selective for fast-type myosin it cannot be applied to muscles with other myosin types.

1.4.3 Blebbistatin

Blebbistatin (a 1-phenyl-2-pyrrolidinone derivative), was identified using a similar screening approach as that for BTS (Straight, Cheung et al. 2003). This small molecule myosin inhibitor is cell permeable and was originally used for inhibition of non-muscle myosin II involved in the cell blebbing process during cytokinesis (Straight, Cheung et al. 2003). Biochemical and kinetic studies of isolated myosins have shown that blebbistatin binds to and stabilizes, the M-ADP- P_i complex with high affinity and interferes with the P_i release. It therefore traps myosin in a state with low actin affinity and blocks the transition of cross-bridges to force-generating states (Kovacs, Toth et al. 2004; Ramamurthy, Yengo et al. 2004). Structural analyses of blebbistatin inhibition using docking simulations and crystallography of *Dictyostelium discoideum* myosin II in a MgADP-vanadate complex have shown that blebbistatin binds to a hydrophobic pocket at the apex of the 50-kDa cleft of myosin, with a stronger affinity to the “closed” state than to the “open” myosin conformation (Kovacs, Toth et al. 2004; Allingham, Smith et al. 2005). These structural results are consistent with a stabilizing effect of blebbistatin on the M-ADP- P_i state.

Blebbistatin has a broader myosin selectivity than BTS and inhibits the actomyosin ATPase activity of several striated muscle and non-muscle myosins in a micromolar concentration range (Straight, Cheung et al. 2003; Kovacs, Toth et al. 2004). Smooth muscle, as well as unconventional myosins such as myosin I, V, X, were reported to be little influenced, with about 100-fold higher half-maximal inhibitory concentration (IC_{50}) (Limouze, Straight et al. 2004). The small effects of blebbistatin on smooth muscle myosin are partly controversial. In a study by Eddinger *et al.* (Eddinger, Meer et al. 2007) it was reported using recombinant smooth muscle heavy meromyosin that blebbistatin can inhibit this myosin type in a low concentration range. The reason for this variance in the biochemical data is not resolved. Blebbistatin can be photoinactivated in the blue wavelength range, which has to be considered when blebbistatin is applied (Sakamoto, Limouze et al. 2005). This property has also been used to photoinactivate the compound in studies on isolated cells and in kinetic analysis of isolated myosins (Ramamurthy, Yengo et al. 2004; Sakamoto, Limouze et al. 2005).

Blebbistatin has been applied in studies of non-muscle myosin II function during cytokinesis and cell migration in different cell types (Straight, Cheung et al. 2003; Ponti, Machacek et al. 2004; Rosenblatt, Cramer et al. 2004; Bastian, Lang et al. 2005). The reported selectivity of blebbistatin for non-muscle myosin over smooth muscle myosin has been used in studies of the relative contribution of these myosin

components during smooth muscle contraction (Ekman, Fagher et al. 2005; Rhee, Ogut et al. 2006). Blebbistatin also inhibits the catch state of permeabilized anterior byssus retractor muscles (ABRM) from *Mytilus edulis* (Butler, Mooers et al. 2006).

Most of our knowledge regarding blebbistatin action has been obtained from biochemical studies of isolated proteins. It is currently unclear if its affinity and action are different in the organized filament lattice of muscle compared to isolated proteins. Before the initiation of this thesis project, no studies of effects of blebbistatin on cardiac and skeletal muscles were available. It was unclear if the myosin selectivity of blebbistatin was present also in muscle tissue expressing the different myosins and if the structural changes and biochemical states observed in isolated proteins corresponded with defined structural states and mechanical effects also in the organized contractile system.

1.5 PURINERGIC RECEPTORS IN CARDIAC MUSCLE

Extracellular nucleotides (ATP, ADP, UTP, UDP) exert a number of physiological functions. In the cardiac muscle, extracellular ATP has been shown to have positive inotropic effects associated with increased cytosolic calcium and increased Ca^{2+} influx through the L-type channels (Vassort 2001). The nucleotides act via purine receptors (purinoceptors), which are divided into two groups: the intrinsic ion channels (P2X receptors) and the G protein-coupled P2Y receptors. The P2X₁, P2X₂, P2X₃, P2X₄, P2X₅ and the P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁ have been found to have cardiac expression (Vassort 2001). Both ATP and UTP can act on P2X receptors and increase contractility via a calcium dependent effect (Froldi, Varani et al. 1997; Erlinge and Burnstock 2008). All five P2Y receptors in the heart are coupled through the G_q receptor to the PLC/IP₃ pathway. Besides this signaling pathway, the P2Y₁₁ receptor also can activate the adenylyl cyclase via G_s, thus increasing cAMP. ATP is a potent agonist of P2Y₂, P2Y₄, P2Y₁₁ receptors, whereas ADP most potently stimulates P2Y₁ (Vassort 2001). Regarding pyrimidines, the unstable nonselective agonist UTP has been shown to have positive inotropic effects on cardiac myocytes via the PLC pathway (Podrasky, Xu et al. 1997). UTP most potently activates P2Y₄ receptor and stimulates P2Y₂ receptor with similar potency as ATP (Nicholas, Watt et al. 1996). However, the receptor selectivity of UTP and UDP is difficult to distinguish in the earlier studies since the unstable UTP rapidly degraded to UDP in the heart by ectonucleotidase (Harden, Lazarowski et al. 1997; Lazarowski, Homolya et al. 1997). To overcome this limitation, stable nucleotide analogues can be used. Techniques to make phosphorothioates which are modified nucleotides like Uridine 5'-O-thiodiphosphate (UDPβS) and uridine 5'-O-3-thiotriphosphate (UTPγS) have been developed by Goody *et al.* (Goody, Eckstein et al. 1972; Lazarowski, Watt et al. 1996). The stable pyrimidine analogues can be used to pharmacologically define the UTP binding P2 receptor subtypes. UTPγS has been reported to be selective for P2Y₂, P2Y₄ receptor and UDPβS is selective to P2Y₆ receptor (Malmsjö, Adner et al. 2000).

Adenine nucleotides (ATP and ADP) are present in the extracellular space of the heart and their release has been studied in different models. It is known that ATP is released together with noradrenaline from sympathetic neurons (Vassort 2001). Other possible sources of ATP include activated platelets, endothelial cells (Ralevic, Milner et al.

1992) and muscle cells during exercise (Forrester 1972; Li, King et al. 2003). In pathological conditions such as hypoxia and hypercapnia, nucleotides levels are reported to increase with a release of ATP from cardiac myocytes and erythrocytes (Williams and Forrester 1983; Bergfeld and Forrester 1992). However, information about sources and conditions of pyrimidine release is limited. A recent study in the pig has shown that UTP is released during cardiac ischemia (Erlinge, Harnek et al. 2005). This suggests that UTP can be released in the heart under pathophysiological conditions. Open questions at the start of the thesis project were whether pyrimidines are released in humans under conditions of cardiac ischemia and which inotropic receptors and cellular signaling pathways are activated by UTP and UDP in the cardiac cells.

1.6 ZEBRAFISH LARVAE AS A MODEL FOR STUDY OF SKELETAL MUSCLE FUNCTION

The recent development in molecular biology and genetics has identified several possible genes involved in human cardiac and skeletal muscle disease. Some of these mutations are identified and currently even forming the basis for specific therapies. Examples are the mutations in the dystrophin gene of patients with Becker and Duchenne muscular dystrophies, where gene or stem cell therapies are attempted (Meregalli, Farini et al. 2008). For other genes in muscle, both the normal function of the protein and possible pathological alterations following mutations are unknown. It is therefore important, of both basic and applied science perspectives, to develop techniques where the functions of several proteins and effects of mutations can be examined in muscle.

Much of our current knowledge about muscle has been obtained from studies of frog and rabbit muscles, which are well suited for both mechanical and structural studies. Recently gene/protein function in skeletal, cardiac and smooth muscle are also studied in transgenic mice. Muscle studies have in addition been performed on other “model” organisms including the fruit fly (*Drosophila melanogaster*, e.g. (Fyrberg, Beall et al. 1991; Maughan, Moore et al. 1998)) and the roundworm (*Caenorhabditis elegans*, e.g. (Moerman and Williams 2006)), which offers specific possibilities in mutational and genetic approach at a significantly lower cost in comparison with the transgenic mouse.

The zebrafish (*Danio rerio*), has been introduced as an important vertebrate model organism for the study of developmental biology and muscle. The advantages of this animal include rapid development, high reproductive capacity, short generation time and transparent embryo and larvae (cf. (Guyon, Steffen et al. 2007)). The zebrafish genome is characterized and equivalents to mammalian and human genes can be identified. Specific mutated zebrafish strains are available and this allows studies of specific gene function in both larvae and adult fish. Moreover, a powerful genetic technique including microinjection of antisense morpholino oligonucleotides can be applied to zebrafish embryos. This enables gene expression to be blocked transiently in a time window of about 1 week during the early developmental larva stage (Nasevicius and Ekker 2000). The morpholino oligonucleotides (morpholino phosphorodiamidate oligonucleotides, MPOs) have a modified ribose in the oligo backbone. These compounds are soluble in water, resistant to a wide range of nucleases and proteases

and have excellent RNA-binding affinity and antisense efficacy. When injected into the zebrafish embryos significant protein depletion can be achieved (Sumanas and Larson 2002).

The zebrafish has a developed musculature and starts to swim within 5 days. The effects of specific gene knock-down by morpholino oligonucleotides on muscle phenotype can therefore be analyzed comparatively rapidly. The calcification of the axial skeleton in the trunk region does not appear until about 7 days (Du, Frenkel et al. 2001). Studies of muscle development in zebrafish have provided insight into the molecular genetic mechanisms that regulate the initial development and differentiation of muscle cells. Two populations of slow and fast muscle precursors have been identified in the developing larvae (Devoto, Melancon et al. 1996). Proteins such as UNC-45, Mef2, obscurin were found to be required for the assembly of thick filaments and sarcomere alignment (Raeker, Su et al. 2006; Hinitz and Hughes 2007; Wohlgemuth, Crawford et al. 2007). Moreover, the zebrafish has been used in the study of molecular mechanisms involved in human muscle diseases. Recently, muscle dystrophy were modeled by depleting dystrophin or dystroglycan in zebrafish, which lead to phenotypes with bent tail, less activity and disruption of muscle fiber structure (Parsons, Campos et al. 2002; Watchko, O'Day et al. 2002). In addition, depletion of the myofibrillar protein, titin, in zebrafish results not only in disrupted skeletal muscle but also in a cardiac myopathy (Seeley, Huang et al. 2007). Most of the previous studies on zebrafish muscle have focused on the genetic, molecular, histological aspects of muscle development. The neural control of the motor activity of zebrafish larvae have been studied by analysis of swimming patterns (McDermid and Drapeau 2006), but functional studies of zebrafish muscle, which might identify subtle and physiologically relevant alterations following gene alterations, are few. The leading idea behind the last part of the thesis was that physiological studies of muscle structure and mechanical properties are required to identify normal and pathological functions of specific proteins in genetically modified zebrafish. Moreover, the techniques for analysis should be developed for the zebrafish larvae, which are directly accessible for future genetic modifications.

2 AIMS

The general aim of the studies in this thesis was to investigate the mechanism of muscle contraction by examining the effects of pharmacological compounds influencing the actin-myosin interaction and/or Ca^{2+} activation systems and by developing physiological measurements of muscle function in the zebrafish larvae model organism.

The specific questions were:

- Does blebbistatin have an inhibitory effect in the organized striated muscle contractile system?
- Does blebbistatin exhibit a myosin selectivity also in the organized contractile system of muscle, similar to that previously reported for isolated proteins?
- Does blebbistatin influence the Ca^{2+} activation systems in cardiac muscle?
- What is the structural state of the blebbistatin bound myosin cross-bridge and the mechanism of the inhibition of force in the organized contractile system of muscle?
- Which inotropic receptors and cellular signaling pathways are activated by UTP and UDP in cardiac cells?
- Can mechanical and structural studies be performed in the zebrafish larvae?
- What are the structural properties of the muscle cells and contractile units in the skeletal muscle of zebrafish larvae?
- What are the mechanical properties of the skeletal muscle in the zebrafish larvae?

3 MATERIALS AND METHODS

The details of the different methods are found in the individual papers. In this section an overview of the techniques is presented.

3.1 OVERVIEW OF ANIMALS, MUSCLE TISSUES AND EXPERIMENTAL APPROACHES

The focus of this thesis is on the regulation of striated muscle contraction. Both cardiac and skeletal striated muscle present a large variety of contractile properties. These differences are certainly obvious between the two main groups of striated muscles (cardiac and skeletal) and also obvious between muscle types within each group. Thus, different striated muscle types from different animals were examined as dictated by the investigative focus in each of my published papers. We have mainly used the mouse (of C57/B16 strain, papers I, II and III), since it is a well established experimental animal and the data and results can be related to a large body of information from normal and genetically modified animals. It should be noted that the cardiac function in mice is different from that in human tissues, e.g. the heart rate of mice at rest is about 450 min^{-1} compared with about 70 min^{-1} in humans. These properties are not only related to the specific properties of ionic channels in the cell-membrane, but also a function of the expression of different myosin isoforms. In paper II, we therefore also included the slower cardiac muscle from a larger animal, pig, which is believed to be physiologically similar to humans. Although we have focused on the analysis of striated muscles of mammals in Papers I-III, we extended the analysis to the striated muscle of another vertebrate, the zebrafish in Paper IV. As presented elsewhere in the thesis, this vertebrate animal has interesting developmental properties and has become a useful novel tool in studying functions of specific genes and proteins. Paper III includes analysis of nucleotide content in human plasma samples and measurements of mRNA (real time PCR) and proteins (Western blot analysis) in human cardiac tissue. These analyses of human material were made by our collaborators at Lund University and we participated in that study with measurements of shortening responses in isolated mouse cardiomyocytes. All animal and human experiments included in the thesis were approved by local ethical committees.

Table 1 summarizes the muscle tissues examined. As stated previously, muscle tissues show a vast array of different contractile properties, which is reflected in the choice of experimental tissues as detailed in the Table. The mouse ventricle is a comparatively fast muscle and contains predominantly the comparatively fast myosin, MHC- α (Krenz, Sadayappan et al. 2007). In paper II where effects of blebbistatin were compared in a range of muscle tissues, we included muscles with different contractile kinetics. Pig atrium and ventricle express MHC- α myosin and, the slower, MHC- β myosin, respectively (Morano, Arndt et al. 1988). Skeletal muscle preparations were obtained from the mouse extensor digitorum longus (EDL, predominantly fast twitch type II (Klueber, Feczko et al. 1989; Moran, Warren et al. 2005)) and from the soleus with a larger fraction type I fibers (Lewis, Parry et al. 1982). In paper II, we also included smooth muscle from the urinary bladder of adult (female, > 8 weeks old) and newborn (1-3 days old, female and male) mice. The urinary bladder of the mouse is a

comparatively fast smooth muscle which expresses the fast splice variant of smooth muscle myosin (cf.(Babu, Celia et al. 2006)). The new-born bladder tissue expresses in addition non-muscle myosins (Morano, Chai et al. 2000; Löfgren, Ekblad et al. 2003). The mechanical properties of the striated dorsal muscles of the zebrafish were examined for the first time in our paper IV. These muscle express fast myosin isoform (Devoto, Melancon et al. 1996).

Table 1: Summary of muscle tissues, preparations, animal species and experiments in the different papers. The relevant Methods sections are indicated within parenthesis.

Muscle tissue	Preparations	Species	Experiments	Papers
Cardiac muscle	Intact papillary (3.2)	Mouse	Force generation (3.5)	I
	Permeabilized muscle (3.2, 3.3)	Mouse Pig	Force generation (3.5) Isotonic quick release (3.6)	I,II
	Isolated cells (3.4)	Mouse	Cell shortening (3.8) Whole cell patch clamp (3.9)	I,III
Skeletal muscle	Permeabilized EDL and soleus (3.2, 3.3)	Mouse	Force generation (3.5) Photolytic inactivation of blebbistatin (3.7) Small angle x-ray diffraction (3.10)	II
	Permeabilized and intact striated muscle (3.3, 3.11)	Zebrafish, larvae and adult	Force generation (3.11,3.12) Small angle x-ray diffraction (3.10)	IV
Smooth muscle	Permeabilized urinary bladder (3.2, 3.3)	Mouse, adult and newborn	Force generation (3.5)	II

3.2 MUSCLE TISSUE PREPARATIONS

Mice were killed by cervical dislocation. Pig hearts were obtained from the local slaughterhouse, immediately perfused with cold cardioplegic solution and transported to the laboratory. Zebrafish were anesthetized with tricaine and killed by rapidly crushing the head (cf. Section 3.11).

The EDL, soleus and urinary bladder preparations from mouse were rapidly excised in physiological salt solution (PSS) containing (in mM) 117.2 NaCl, 25.2 NaHCO₃, 4.7 KCl, 1.2 MgCl₂, 1.2 KH₂PO₄, 2.5 CaCl₂, 11.1 glucose gassed with 5% CO₂/95%O₂ at room temperature. The urothelium was gently removed from the adult urinary bladders.

Thin trabecular preparations of the mouse and pig cardiac ventricles were dissected in cardioplegic solution containing (in mM): 120 NaCl, 16 KCl, 16 MgCl₂, and 1.2 CaCl₂, 5 Tris-buffer (pH 7.4), with 3.5% Dextran 40. These striated and smooth muscle tissues were permeabilized (3.3) for further mechanical and structural experiments as described below (3.5-3.6). For preparation of intact papillary muscles from the mouse, the heart was dissected and gently massaged to empty remaining blood in the ventricles. Two papillary muscles of left ventricle were dissected and tied with silk thread at both ends for recording of isometric force (3.5). The preparation of zebrafish muscles is described below (3.11)

3.3 PERMEABILIZED MUSCLE PREPARATIONS

Membrane permeabilization ('skinning') is a method whereby the cellular membranes are permeabilized or removed. This offers the technical advantage of keeping the contractile machinery essentially unchanged and allowing contraction and relaxation to occur under complete and strict control of intracellular environment. Permeabilization of the membranes are commonly achieved by applying the detergent Triton-X-100 and/or Ca²⁺-chelator EGTA, followed by freeze-glycerination (treatment with glycerol at -20°C) (cf.(Arner and Hellstrand 1985; Morano, Rosch et al. 1990; Balogh, Li et al. 2005)). The preparations can be stored in a solution with 50% glycerol, for several weeks at -20°C. The methods for cardiac, skeletal and smooth muscles of pig and mouse are presented in more detail in the respective papers. For zebrafish striated muscles, where no information on skinning procedures was available, we used permeabilization with a low concentration of Triton X-100 (0.1% for 3 hours and room temperature). Because of the destroyed cell membrane, low molecular components of the myoplasm are lost. Thus a solution that mimics the intracellular environment and includes relevant concentrations of ATP, phosphocreatine, Mg²⁺, K⁺, pH buffers and reducing agent is used. In striated muscle, the muscle preparations can be considered relaxed at free Ca²⁺ concentrations below 10 nM (pCa>8, where pCa is the negative logarithm of the concentration of free Ca²⁺ expressed in mol/L). At a free Ca²⁺ concentration of about 50 μM (pCa 4.3) the muscles are maximally activated. The free [Ca²⁺] can be varied by altering the ratio CaEGTA/EGTA in the solutions.

In study I, II, IV, the maximal active force and the maximal shortening velocity of permeabilized muscle preparations were determined following activation at pCa=4.3, using methods described in sections 3.5 and 3.6. In study I, the Ca²⁺-sensitivity of active force was determined by exposing preparations to increasing free [Ca²⁺]. In the absence of ATP, myosin and actin in striated muscle form rigor complexes independently of the [Ca²⁺]. The rigor state is a well defined structural and biochemical state in the cross-bridge cycle (cf. Figure 1). Rigor was obtained in study II in the muscle preparations by omitting ATP in the bathing solutions. In paper II the influence of inorganic phosphate (P_i) was examined to study the effects of blebbistatin on the force generating reaction, which is considered to be coupled to the P_i release. The P_i induced inhibition of force was examined by exposing the preparation to different concentrations of P_i at the plateau of maximal contraction.

In smooth muscle, the activation of contraction is initiated by a Ca²⁺ dependent phosphorylation of the regulatory myosin light chains. This is an ATP dependent process catalyzed by the enzyme myosin light chain kinase. Since this process might be

affected by the examined compound (e.g. blebbistatin) and indirectly influence the force responses, we used smooth muscle preparations where the regulatory light chains were maximally (thio)phosphorylated using ATP γ S as substrate for the Ca²⁺ dependent phosphorylation (Arheden, Arner et al. 1988; Jaworowski, Ozturk et al. 1999).

3.4 MOUSE CARDIOMYOCYTES

Isolated mouse cardiomyocytes were used in cell shortening experiments (section 3.8) and in electrophysiological studies (whole cell patch clamp, section 3.9) for determining effects of blebbistatin and nucleotides (papers I and III). The cells were dissociated with collagenase and isolated according to a standard protocol (<http://www.signaling-gateway.org>; Procedure Protocol ID: PP00000125). The mouse was killed and the heart was rapidly removed from the chest cavity and immediately perfused through aorta with a calcium-free perfusion buffer containing (in mM) 113 NaCl, 4.7 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2 MgSO₄, 12 NaHCO₃, 10 KHCO₃, 10 HEPES, 30 taurine, 5.5 glucose, 10 BDM, pH 7.4 at 37°C for 5 min followed by perfusion with low Ca²⁺ (12.5 μ M) perfusion buffer with 571 U/ml collagenase for 15 min. When the heart appeared swollen and pale, the ventricles were removed from the perfusion system and gently teased to smaller pieces. Digestion was stopped by adding 5% calf serum. Thereafter, Ca²⁺ was reintroduced to the cells in several steps from 12.5 μ M to a final concentration of 1 mM. The cells were used within 24 hours.

3.5 ISOMETRIC FORCE

Isometric force recordings were used to determine the effects of blebbistatin on the force generating actin-myosin interactions in different muscles (papers I and II). Force measurements on zebrafish muscles are described separately (3.11). In principle, the preparations were mounted between a fixed pin and a pin attached to an AE801 force transducer. The length of the preparations could be adjusted using a micrometer screw. The force signal was A/D converted (sampling frequency 0.4-10 kHz) and recorded using AD-instruments Chart software. Small aluminum clips at the ends of the skinned preparations were used to attach the muscles to the fixed pin and the transducer. For single muscle fibers of EDL and soleus, cellulose acetate was used to attach the fibers between carbon rods on the micromanipulator and the force transducer. The skinned preparations were mounted at room temperature in solution as described above in small Perspex baths or solution bubbles (~ 200 μ l) with continuous stirring using miniature magnetic stirrer rods. The intact papillary muscles were mounted in PSS at room temperature in Perspex baths and activated using electrical field stimulation (0.5 ms pulses, 1 Hz). The stimulation voltage was set to a level about 10% above that giving maximal contractile response (supramaximal voltage).

3.6 ISOTONIC QUICK RELEASE

The maximal shortening velocity (V_{\max}) was determined using the isotonic quick release method as previously described (cf. (Arner and Hellstrand 1985)). V_{\max} is considered to be determined by the rate of cross-bridge detachment and this technique was therefore used in Study I to examine the effects of blebbistatin on cross-bridge detachment reactions in the mouse cardiac muscle. A permeabilized mouse cardiac muscle preparation was mounted at room temperature at a low passive tension between a force transducer and an isotonic lever. Using a spring, different afterloads could be

applied to the lever. The preparations were maximally activated with Ca^{2+} containing solution (pCa 4.3). When a stable contraction was established, a series of releases with different afterloads was imposed. For each release, the afterload and shortening at 100 ms after release were measured. The V_{\max} is determined by fitting a hyperbolic equation to the force and velocity data and then extrapolating to zero force. In each preparation, force-velocity relationships were first determined under control, maximally activated, conditions. Then the muscles were relaxed and activated in the absence or presence 1 μM of blebbistatin. For comparison with a condition with low cross-bridge detachment rate, we included measurements in a low ATP/high ADP solution.

3.7 PHOTOLYTIC INACTIVATION OF BLEBBISTATIN

Blebbistatin is sensitive to blue light (Sakamoto, Limouze et al. 2005), a quality that previously has been used to reverse its inhibitory effects in cells (Sakamoto, Limouze et al. 2005; Farman, Tachampa et al. 2007). In study II, we attempted to rapidly reverse the inhibitory effect of blebbistatin in striated muscles using a millisecond light flash from a Xenon flash lamp with a high intensity in the blue light wavelength region (Arner, Goody et al. 1987; Rapp and Guth 1988). Permeabilized preparations of EDL and soleus were maximally Ca^{2+} -activated in the presence of blebbistatin, at a concentration inhibiting force to about 2 % of the initial. Prior to photolysis, the preparations were transferred to a small cuvette (volume 50 μl) equipped with a quartz window and illuminated. Several light flashes were applied and the force transients after flashes were recorded.

3.8 CARDIOMYOCYTE SHORTENING

The effects of blebbistatin and nucleotides on cardiomyocyte shortening were examined in studies I and III. In these experiments, the isolated cardiac myocytes were kept in a cuvette on the stage of an inverted microscope. The cuvette was perfused with Ca^{2+} containing oxygenated HEPES buffered salt solution contained (in mM): 150 NaCl, 5.4 KCl, 10 HEPES, 2 MgCl_2 , 10 glucose and 1.5-1.8 CaCl_2 , pH 7.4 at room temperature. Contractions were elicited using electrical field stimulation at 0.5-1 Hz (0.5-4 ms pulse duration, supramaximal voltage). The cells were visualized via a glass window in the cuvette and the shortening responses were recorded using a CCD camera system and a frame grabbing unit (with a video frame rate of 25 Hz). Relative cell shortening was compared before and after addition of chemical compound and expressed as percentage of the responses recorded in the absence of compounds.

3.9 WHOLE CELL PATCH CLAMP

In study I, the effects of blebbistatin on action potentials and Ca^{2+} -currents (I_{Ca}) of isolated mouse cardiac myocytes were examined using the whole cell patch clamp technique. The isolated cells were allowed to settle on the bottom glass of a perfusion cuvette on the stage of an inverted microscope and were perfused with HEPES buffered salt solution (pH 7.4) at room temperature. In principle, the cells are attached to microelectrodes as shown schematically in Figure 2. The micropipette is used both for electrode voltage-recording and current-injection. A freshly made glass pipette with a small smooth opening is gently pressed against the myocyte membrane. Gentle suction in the patch pipette causes a deformation of the myocyte membrane and a tight "Giga-

ohm” seal is established. When the patch membrane in the pipette disrupted with a stronger suction, cytoplasm and pipette solution start to mix (Fig. 2). This enables us to control the environments of both sides of the cell membrane. To record action potentials, the pipette solution contained (in mM): 150 KCl, 10 HEPES, 5 MgCl₂, pH 7.2, while the bathing solution contained (in mM): 150 NaCl, 5.4 KCl, 10 HEPES, 2 MgCl₂, 10 glucose, 1.5 CaCl₂, pH 7.4. Action potentials were elicited using a 3-ms current injection. The duration of the action potential was measured at different repolarization levels and was compared before and after addition of blebbistatin. A voltage clamp technique was used for recording of the I_{Ca} . In voltage clamp experiments, the membrane potential is maintained constant, while the current flowing through the membrane is measured. When membrane potential reaches a certain level, it causes opening of the voltage-gated channels. In our experiments, an initial holding potential of -40 mV was used and I_{Ca} is elicited by giving a series of steps of different amplitude from -40 to 40 mV. It is important to prevent K⁺ and Na⁺ currents while recording the Ca²⁺ current. Therefore, Na⁺-ions are omitted in the bathing solution and tetraethylammonium (TEA, 135 mM) and 4-aminopyridine (4-AP, 15 mM), general inhibitors of K⁺-channels, were included. Also, the patch pipette solution contained the K⁺-channel blocker, Cs⁺, instead of K⁺. I_{Ca} properties are described by the voltage-current relationship and current inactivation kinetic analysis. The decay of I_{Ca} with time (t) was fitted by the sum of two exponential components with amplitudes A and time constants τ using the following formula: $I_{Ca} = A_{fast} \times \exp(-t/\tau_{fast}) + A_{slow} \times \exp(-t/\tau_{slow})$.

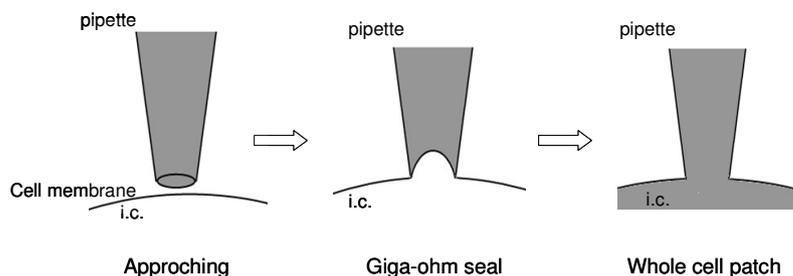


Figure 2. Schematic drawing of the whole cell patch clamp technique. The pipette electrode is approaching cell membrane (left). Application of light suction results in formation of a Giga-ohm seal (middle). The patch is ruptured by additional suction and pipette solution mixes with cytoplasm (right). i.c. (intracellular).

3.10 SMALL ANGLE X-RAY DIFFRACTION

Small angle x-ray diffraction provides information of muscle ultrastructure using diffraction from the periodic and regular structures of the myofilaments. The regular and periodic arrangement of the myofilaments in muscle causes x-ray light scattering that can be analyzed and give information on e.g. distances between myofilaments (filament spacing), periodicities along the filaments (e.g. cross-bridge periodicity) or mass transfer between the filaments (e.g. attachment and movement of cross-bridges). The experiments in study II and IV were performed using synchrotron radiation at beamline A-2 at HASY-lab, DESY, Hamburg, Germany. This is a dedicated beamline for small angle scattering experiments of e.g. polymer structures like muscle. In the DESY-facility, positrons are accelerated at near light speed in a vacuum tube system.

The x-ray light is obtained from the synchrotron radiation which is generated when the high speed positrons are diverted from their track by bending magnets. The light is positioned on the muscle preparations and the regularly arranged filaments, with nanometer distances, scatter the light and create diffraction patterns. These are recorded by a detector, usually placed 2-3 m away from the diffraction source in order to obtain sufficient resolution. Typically vertebrate muscles have equatorial and meridional reflections as shown schematically in panel A of Figure 3 (for a review see (Haselgrove 1983)). The regular and helical structures along the length of the muscle fiber give meridional reflections and the layer lines. The layer lines are a series of lines essentially in parallel to the equator positioned at the sides of the meridian. Examples are the strong 14.3 nm meridional reflection from the myosin head projections along the thick filaments and the layer lines from the actin filaments and helical myosin projections in relaxed muscle. In the direction perpendicular to the muscle long-axis, the lattice of the double hexagonal array of thick and thin filaments (Panel B of Figure 3) produces equatorial reflections, which appear as separate spots, 1.0 and 1.1 as shown in panel C of Figure 3. The spacing of the 1.0 and 1.1 reflections (d_{10} and d_{11}) are inversely related to the distance between filaments (Millman 1998). A wider filament spacing results in 1.1 and 1.0 reflections closer to the origin. The lattice spacing is dependent on sarcomere length (SL), the lattice volume (reflecting the volume of a sarcomere) can be calculated as lattice area times sarcomere length (lattice volume = $(2/\sqrt{3})d_{10}^2 \times SL$). The 1.1 and 1.0 intensities are influenced by the position of the myosin heads. The relative intensity of the equatorial 1.1 and 1.0 reflections provides information of the distribution of mass between the filaments. When myosin heads attach to actin during contraction or rigor, the intensities of the reflections change, with a decrease in 1.0 and an increase in 1.1 intensity.

The small angle x-ray diffraction technique was applied to permeabilized preparations of EDL to examine the structural effects of blebbistatin in study II and to examine the structure of relaxed and contracted adult and larval zebrafish muscle (study IV). The preparations were mounted at room temperature horizontally between a fixed pin and a force transducer in a 200 μ l trough made from a folded Kapton membrane. For stimulation of intact zebrafish larvae muscles the bath was equipped with platinum wires for electrical field stimulation. Diffraction images were recorded using a CCD-camera based system. In study II, the effects of blebbistatin on the mass transfer between filaments and on the relaxed layer line patterns was determined in permeabilized EDL muscles. In study IV the filament orientation, the filament spacing at different sarcomere lengths and the contraction induced mass transfer between filaments were recorded in intact zebrafish larvae and in permeabilized adult zebrafish muscles.

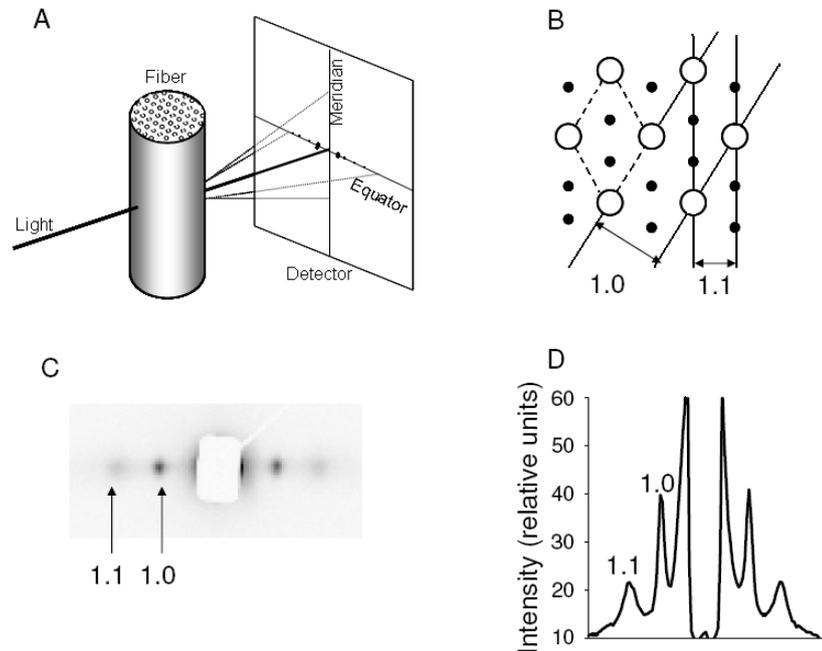


Figure 3. Small angle x-ray diffraction of striated muscle. Panel A shows schematically the relation between the equatorial and meridional reflections and muscle fiber structure (Modified from (Squire and Knupp 2005)). Panel B A model showing the hexagonal arrangement of myosin (open circle) and actin (filled circle) and the origin of the 1.0 and 1.1 reflections. Dotted lines shows unit cell of filament lattice area (Millman 1998). Panel C shows an original detector image of the equatorial peaks (from study II) from a relaxed EDL muscle preparation and Panel D the corresponding scan of the intensities.

3.11 ZEBRAFISH LARVAE PREPARATION AND MOUNTING

In study IV, we developed a novel method to examine mechanical function and structure of skeletal muscle from zebrafish early larvae. The study was performed in 5-7 days larvae, which is the time point when the preparations can be influenced by a morpholino antisense approach. The animals were anesthetized by transferring to the E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl₂ and 0.16 mM MgSO₄) containing 0.017% of the anesthetic compound tricaine. The heads were rapidly crushed and the preparations were mounted as shown in Figure 4. The two corners of the aluminum clips were folded at 45° angles to trap the head and the tail parts of the larva. In addition to the main muscular part, the abdominal part of the larva was usually kept in order to minimize mechanical disruption of the preparations. Small holes were made in the aluminum foil for firmly attaching the preparation at room temperature (~22°C) in a cuvette (Panel B, Figure 4) between a pin and the modified arm of an AE801 force transducer. The length of the preparation was adjusted with a micrometer screw. The bath had a volume of about 0.5 ml and was perfused with solution as described

below at room temperature. The cuvette was also equipped with two platinum electrodes for electrical field stimulation and a glass window for observation of the preparation and sarcomere length using an inverted microscope. For the small angle x-ray diffraction experiment, the preparations were mounted similarly in the cuvette described in section 3.10.

The force signal was recorded using an AD-converter at high sampling rate (10 kHz). The experiments were performed in a modified Krebs-Henseleit solution containing (in mM) 117.2 NaCl, 25.2 NaHCO₃, 4.7 KCl, 1.2 MgCl₂, 1.2 KH₂PO₄, 2.5 CaCl₂, 11.1 glucose gassed with 5% CO₂/95%O₂. We found that this solution resulted in more reproducible single twitch contractions of the larva than the E3 medium or frog Ringer solution. In the x-ray diffraction experiments, when the cuvette could not be perfused, the Krebs-Henseleit solution was replaced by a MOPS buffered solution of the following composition (mM): 118 NaCl, 24 MOPS, 5 KCl, 1.2 MgCl₂, 1.2 Na₂HPO₄, 1.6 CaCl₂, 10 glucose (pH 7.4) giving similar contractile responses.

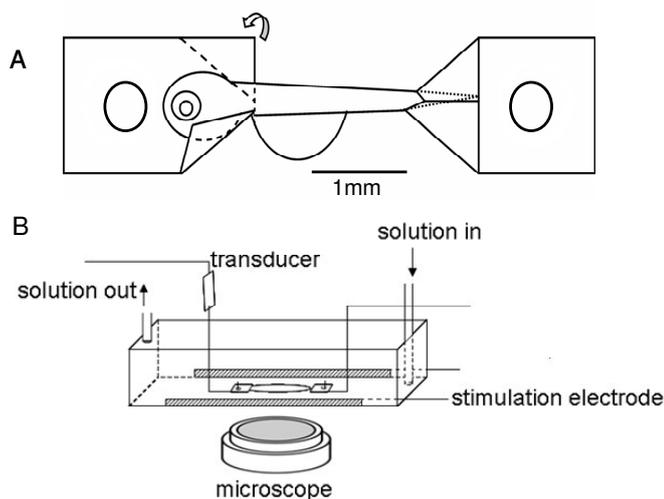


Figure 4. Panel A shows schematically the method for mounting the zebrafish larvae muscle preparation between two aluminum clips. Panel B shows schematically the experimental bath on the inverted microscope. Illustration modified from paper IV.

3.12 MECHANICAL EXPERIMENTS ON ZEBRAFISH LARVAE

The larvae preparations could be reproducibly activated using electrical field stimulation. Single twitch contractions were activated using 0.5 ms pulses at supramaximal voltage. To determine force-length relationship, single twitches contractions were elicited with 2 min intervals and the preparations were stretched to a new length at each interval. Single twitch force and sarcomere length were recorded for each contraction. The optimal length was defined as the sarcomere length giving maximal active single twitch force. To determine the force-stimulation frequency relationship, the preparations were stretched to optimal length and stimulated with 220

ms stimulation trains at an increasing frequency from 20 to 210 Hz with an 8 min interval in between trains. Muscle fatigue properties were determined using repeated 220 ms tetani at 186 Hz. The stimulations started with stimulation at an 8 min interval. The train rate was gradually shortened (0.0042, 0.014, 0.032, 0.054, 0.08, 0.127 and 0.3 s⁻¹) and the tetanus force responses were analyzed when the amplitude of force became reproducible at each train rate. After exposure to the highest train rate, the muscles were allowed to recover for 8 minutes and again stimulated to determine the recovery after the fatigue protocol. To explore if the larvae muscle contractions were due to fast or slow twitch myosins we performed experiments using BTS which is considered to be an inhibitor of myosin II, with a selectivity for fast myosin (Cheung, Dantzig et al. 2002).

4 RESULTS AND DISCUSSION

4.1 INHIBITION OF MUSCLE CONTRACTION BY INHIBITING ACTIN-MYOSIN INTERACTION

As outlined in the Introduction, contraction at the actin-myosin interaction level consists of several steps, all of them under the possible control of a host of intracellular and extracellular regulatory mechanisms. Our first focus in this investigation was therefore on factors directly interfering with actin-myosin interaction. Within the action spectrum of blebbistatin (and BTS), the following factors were considered important: (i) linking up biochemical actin-myosin binding studies with physiological contraction studies; (ii) the different affinities for different myosin isoforms (Shaw, Ostap et al. 2003; Limouze, Straight et al. 2004). Since both striated and smooth muscle tissues comprise different myosin isoforms with potentially different functions, compounds with myosin selectivity can be used to differentiate the relative roles of the myosin. Moreover, a selective inhibitor of contraction can be potentially useful in experiments where, e.g. metabolism, cell signaling or membrane properties, are examined.

The application of blebbistatin to study of actin-myosin interaction in muscle fiber preparations requires that the potential effects on regulatory systems are considered and in papers I and II, we therefore examined if blebbistatin interferes with Ca^{2+} regulation in the cardiac muscle cells (discussed in Section 4.2). The cross-bridge reactions in muscle are influenced by the filament organization and possible mechanical or structural constraints present within and between the filaments of the organized muscle tissue (Goldman 1987). In paper II, we therefore related the sensitivity of contractile activation in different muscle systems with the different predominant myosin isoform in each muscle and compared with previously obtained data (Section 4.3). The structural effects and the mechanism of force inhibition were also considered (Sections 4.4 and 4.5).

4.2 EFFECTS OF BLEBBISTATIN ON Ca^{2+} -ACTIVATION SYSTEMS IN CARDIAC MUSCLE

Blebbistatin is found to be an inhibitor of interaction between actin and myosin. This is clearly demonstrated in studies using isolated contractile proteins (Kovacs, Toth et al. 2004; Limouze, Straight et al. 2004; Ramamurthy, Yengo et al. 2004). However, if this compound is to be used also in the more complex system of intact muscle, effects on activation systems have to be considered. Previously, another substance, BDM, proposed to interfere with the interaction between actin and myosin (Blanchard, Smith et al. 1990) was later shown also to influence Ca^{2+} -activation of both striated (Gwathmey, Hajjar et al. 1991; Siegman, Mooers et al. 1994) and smooth muscles (Lang and Paul 1991; Österman, Arner et al. 1993). To date, such possible “unwanted” effects of blebbistatin upstream from the interaction between actin and myosin had not been investigated. Therefore in paper I, we examined the effects of blebbistatin on cardiac muscle and its effects on Ca^{2+} influx and thin filament regulation. Blebbistatin promptly inhibited contraction of intact papillary muscles, isolated cells and skinned trabecular muscles (Paper I). Since the skinned preparations were examined at constant Ca^{2+} levels, we can conclude that the inhibition is not primarily due to effects on the

levels of activator Ca^{2+} . To further examine potential effects of blebbistatin on Ca^{2+} -influx, we performed electrophysiological measurements under conditions where contractions were significantly inhibited (10 μM blebbistatin for 5 minutes when cellular shortening responses were about 20% of control). Under these conditions, the duration of the action potential (fig. 4, paper I) was not altered. Using a voltage clamp approach, we also showed that the current-voltage relationship, as well as the time constants and amplitudes of the current decay of the L-type Ca^{2+} channels were not influenced by 10 μM blebbistatin which otherwise resulted in significant attenuation of active force (fig. 4, paper I). Experiments on skinned trabecular preparations from the mouse heart showed that the Ca^{2+} -sensitivity was unaltered by a concentration of blebbistatin giving a significant inhibition of active force (see also further comments below in this section). Our conclusion is thus as follows: the attenuation of contractile responses in cardiac tissues by blebbistatin does not involve effects on Ca^{2+} -influx but is the result of a specific interference with the interaction between myosin and actin. During the revision of paper I, a new publication confirmed the absence of blebbistatin's effects on Ca^{2+} -transients and other electrophysiological parameters on rabbit hearts (Fedorov, Lozinsky et al. 2007). These results from rabbit and mouse were later also confirmed for rat cardiac tissue (Farman, Tachampa et al. 2007). Blebbistatin is thus an effective and specific inhibitor of the contractile system in cardiac muscle without effects on the excitation-contraction coupling.

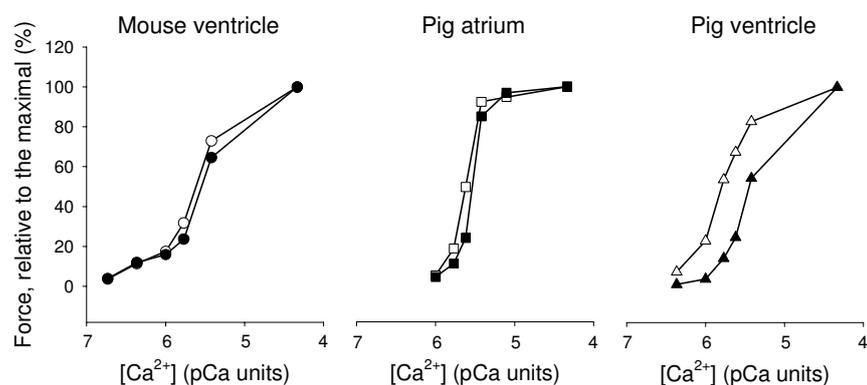


Figure 5. Force vs $[\text{Ca}^{2+}]$ for permeabilized cardiac preparations of mouse ventricle (circles), pig atrium (squares) and pig ventricle (triangles) in control medium (open) and in the presence of 3 μM blebbistatin (filled symbols). Force values are normalized to maximal force at pCa 4.3. Data were compiled from fig. 3 of paper I and fig. 5 of paper II and show mean values only. The force (at pCa 4.3) in 3 μM blebbistatin was 39%, 48% and 67% of the initial in the mouse ventricle, pig atrium and pig ventricle respectively.

In paper I, we examined the Ca^{2+} -sensitivity of permeabilized cardiac muscle preparations and concluded that the inhibitory effects of blebbistatin on force are not dependent on effects on thin filament regulation. In mouse cardiac preparations, 3 μM blebbistatin did not alter the Ca^{2+} -force relationship although the force was inhibited by about 60% (fig. 3, paper I, Fig. 5 above). We also noted that with higher concentrations

of blebbistatin (10 μM), the Ca^{2+} -sensitivity of the mouse ventricular preparations was slightly lower, but that this effect was difficult to determine due to the low absolute forces at this blebbistatin concentration (fig. 3, paper I). In paper II, we extended this analysis of Ca^{2+} -sensitivity by including skinned preparations from atrium and ventricle of pig hearts (fig. 5, paper II). Blebbistatin (3 μM) attenuated active force in both atrium and ventricle (respectively, about 50 and 70% of maximal activation remaining). The Ca^{2+} -force relationship of atrium preparations was unaffected, whereas it was significantly shifted towards higher Ca^{2+} concentrations in the ventricle preparations (fig. 5, paper II, Fig. 5 above).

It is currently not possible to exclude a direct effect of blebbistatin on the pig ventricular troponin/tropomyosin complex which can explain the effects of Ca^{2+} -sensitivity. However, the absence of effects on thin filament based Ca^{2+} -sensitivity regulation in other cardiac muscle types, argues against such an explanation. Rather, blebbistatin affects Ca^{2+} -sensitivity in the ventricular cardiac muscle by interfering with cross-bridge attachment. We propose therefore the following series of events to explain this altered Ca^{2+} -sensitivity. It is well known that attachment of cross-bridges in a strong binding configuration in striated muscle affects the position of tropomyosin and the Ca^{2+} -sensitivity of the troponin system (Bremel and Weber 1972; Güth and Potter 1987). One example of this is the activation of contraction induced by strong rigor attachment (e.g. the rigor contraction in fig. 1, paper II). In cardiac muscle, this potentiation is considered to be a part of the activation: cross-bridge attachment (initiated by increased cytoplasmic Ca^{2+}) leads to strong binding states and an increase in the Ca^{2+} -sensitivity of the system (Gordon, Homsher et al. 2000). Our results show that the blebbistatin has minor effects on the Ca^{2+} -sensitivity in comparatively fast cardiac muscles (fast cross-bridge cycling, mouse ventricle and pig atrium) but pronounced effects in slower cardiac muscle (slow cross-bridge cycling, pig ventricle). Thus, by removing cycling cross-bridges, blebbistatin alters Ca^{2+} -sensitivity by a proportionally greater reduction of the number of strongly attached cross-bridges in slow muscles. It is possible that this reflects a difference between the distribution of the various cross-bridge states at any particular moment of the contraction: slower ventricular muscle (high β -myosin content) has a distribution of largely strongly attached cross-bridge states able to influence the thin filament system. This also suggests that the attachment induced effects on Ca^{2+} -sensitivity, possibly also length-dependent effects (Frank-Starling mechanism), would be stronger in slower cardiac muscle, i.e. in ventricle vs. atrium and in cardiac muscle of larger animals vs. smaller.

4.3 MYOSIN SELECTIVITY OF BLEBBISTATIN IN THE ORGANIZED CONTRACTILE SYSTEM

Blebbistatin has been reported to inhibit actin-myosin ATPase of several striated and non-muscle myosins (Limouze, Straight et al. 2004). It inhibited actin-activated ATPase of myosin with inhibition constants clearly falling in three groups (Figure 6): low micromolar (skeletal, non-muscle IIA & IIB, cardiac), high micromolar (smooth muscle) and submillimolar (unconventional myosins) (Straight, Cheung et al. 2003; Limouze, Straight et al. 2004). In paper II, we examined if this myosin selectivity also was present in the organized contractile system of muscle. We used a range of muscles with different contractile kinetics, which have been found to correlate with expression

of myosin isoforms, from the comparatively fast mouse EDL, via the slower soleus and cardiac muscles to the smooth muscle of the mouse urinary bladder.

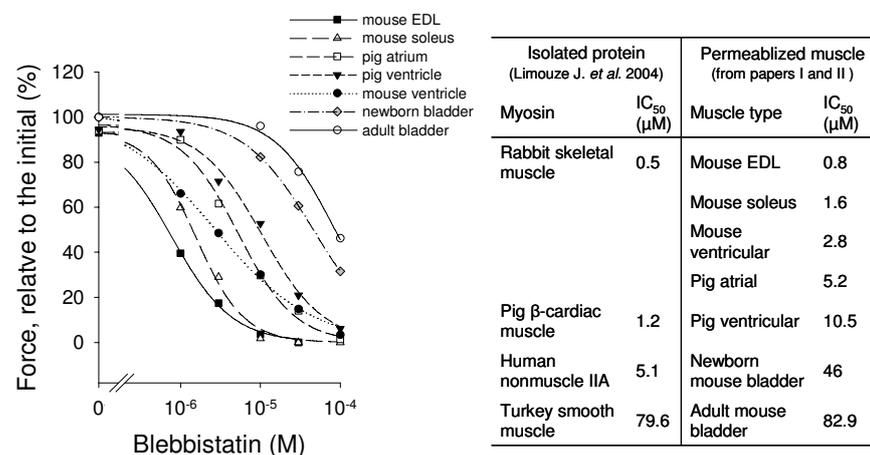


Figure 6. Left panel: Concentration-dependence of inhibition of force in different permeabilized muscle preparations. Data were compiled from fig. 4 of paper II and show mean values only. Data from mouse ventricular fibers examined in paper I are also included. Right panel: shows a table of IC₅₀ values derived from data in papers II and I.

Blebbistatin inhibited the active contraction of all striated muscle preparations in a time scale of minutes (fig. 1, paper I; fig. 4, paper II). The relaxant effect was concentration dependent (fig. 4, paper II). To obtain an estimate of the IC₅₀ values in the different striated muscles, we used an incubation time of 20 minutes at room temperature and examined the force in two contractions before and after exposure to blebbistatin. This was in part a compromise, since we could not confirm that blebbistatin binding was at steady state under those conditions (cf panels A-C fig 4, paper II). Alternatives would be to keep the muscles activated for a longer time period during exposure to blebbistatin or using longer incubation times, possibly under cold conditions (as used in (Farman, Tachampa et al. 2007)). We avoided these approaches since prolonged contractions might affect stability of the striated muscle fiber preparation and since low temperature conditions can alter the relaxed myosin configuration (Xu, Offer et al. 2003; Xu, Gu et al. 2006) towards a state with lower blebbistatin binding affinity (Kovacs, Toth et al. 2004). It has been shown that blebbistatin does not affect the myosin light chain phosphorylation in smooth muscle (Rhee, Ogut et al. 2006), but to avoid possible interference with the dynamic processes of light chain phosphorylation and spurious changes of the activation level and contraction, we thiophosphorylated the light chains, ensuring constant and maximal activation (see Methods). Figure 6 derived from data in paper II, shows the concentration-dependent blebbistatin attenuation of force in the different muscle types. A large range in blebbistatin sensitivity was observed, with the fast skeletal muscles (EDL) being the most sensitive and the smooth muscle the least. The Table in the right panel of Figure 6 compares our IC₅₀ values for force attenuation with Limouze's corresponding data on myosin ATPase (Limouze, Straight et al. 2004). Overall, the data show a marked correlation between the effects of

blebbistatin in both *in vitro* situations: on the one hand myosin ATPase (isolated proteins in solution) and on the other hand active force (filamentous *in situ* organization). The authors are aware of the difficulties that exist in directly “translating” ATPase activity to force development, but blebbistatin's attenuating effect on active force depends on muscle type, an observation correlated to the biochemical assays. The reason for this selectivity of blebbistatin is not completely understood but it may reflect the myosin structure and amino acid sequence in the blebbistatin binding site (Allingham, Smith et al. 2005). It should also be noted, as discussed further below, that the blebbistatin sensitivity might also reflect the relative population of cross-bridge states available for blebbistatin binding. In general, the blebbistatin sensitivity of different muscle types is weakly related to their maximal shortening velocity (V_{max}). V_{max} values of different muscles has previously been reported (EDL 5.9 muscle lengths (ML)/s, mouse soleus 2 ML/s, (Crow and Kushmerick 1983), pig atrium 4 ML/s, pig ventricle 2.5 ML/s, (Svensson, Morano et al. 1997), smooth muscle 0.17 ML/s, (Sjuve, Arner et al. 1998)). The faster muscles have a comparatively high affinity for blebbistatin, whereas slower muscles have lower affinity for blebbistatin. This correlation may thus relate to the relative population of blebbistatin binding cross-bridge states in fast and slow muscle types.

In the original biochemical studies it was observed that actin-activated ATPase of turkey gizzard myosin HMM was less inhibited by blebbistatin than the striated and non-muscle myosins (Straight, Cheung et al. 2003; Limouze, Straight et al. 2004). Since contraction of some smooth muscle types has been found to result from cross-bridge interaction from both smooth and non-muscle myosins (Morano, Chai et al. 2000; Löfgren, Ekblad et al. 2003; Rhee, Ogut et al. 2006), blebbistatin has been used as a tool to differentiate between the relative contribution of these two contractile components in newborn and adult smooth muscles (Ekman, Fagher et al. 2005; Rhee, Ogut et al. 2006). However, the finding of less sensitivity of blebbistatin on smooth muscle myosin was challenged by Eddinger *et al.*, who reported more prominent blebbistatin effects for recombinant smooth muscle HMM fragments and for tonic force of rabbit renal and saphenous arteries (Eddinger, Meer et al. 2007). Our data showed the IC_{50} of blebbistatin for maximally activated adult urinary bladder at 22°C was significantly higher than that of striated muscle and would be more consistent with previously reported for turkey gizzard HMM. Our results from newborn tissue, which contain a mixture of smooth and non-muscle myosin, are also consistent with a stronger effect of blebbistatin on non-muscle myosin compared with smooth muscle myosin. The reason for the differences in biochemical data (turkey gizzard HMM and recombinant HMM) and in muscle fiber data from different tissues (urinary bladder vs rabbit renal and saphenous arteries) is unknown. It is possible that the structure and properties of the myosin components influence the biochemical assays. The experimental conditions, for instance different temperature or the level of activation might affect the population of blebbistatin binding cross-bridge states in smooth muscle tissue preparations and result in different inhibitory effects.

4.4 STRUCTURE OF BLEBBISTATIN-MYOSIN CROSS-BRIDGE

Blebbistatin is proposed to bind to the 50-kD cleft region of myosin (Allingham, Smith et al. 2005) and thus exert its influence on the actin-myosin interaction. The possibility

of accompanying structural effects of such a binding in muscle fibers had not been investigated. We therefore performed a structural analysis of the filamentous *in situ* myosin molecule in the presence and absence of blebbistatin using small x-ray diffraction of muscle fibers. The experiments were performed in permeabilized mouse EDL preparations. Original records of the equatorial patterns are shown in fig. 2 of paper II. To answer the question whether cross-bridges, in the presence of blebbistatin bound to myosin, were either associated with or dissociated from actin, intensities of the equatorial 1.0 and 1.1 reflections were analyzed in muscles with the presence 30 μM blebbistatin. In the absence of blebbistatin the 1.1/1.0 ratio was low in the relaxed state and increased during contraction (relaxed: 0.46 ± 0.02 ; contracted: 2.99 ± 0.60 ; fig. 2, paper II). This reflects the change in equatorial intensities are due to mass transfer from thick to thin filaments during cross-bridge interaction (Matsubara, Maughan et al. 1989). In the presence of blebbistatin, which fully inhibited contractions, the equatorial 1.1/1.0 ratio remained similar to that of the relaxed muscle at both low and high $[\text{Ca}^{2+}]$, (relaxed: 0.36 ± 0.03 , contracted: 0.46 ± 0.14 ; fig. 2, paper II). This shows that the mass transfer to the thin filaments in Ca^{2+} -containing solution is inhibited by blebbistatin. Thus blebbistatin traps myosin in state dissociated from actin and thereby prevents cross-bridges from attaching and entering into force generating reactions.

We also found that myosin layer lines were visible in the presence of blebbistatin in solutions containing low or high free calcium (data not shown). The layer lines are considered to originate from the helical arrangement of myosin heads on the thick filaments (Haselgrove and Rodger 1980). The data thus suggest that the dissociated myosin head with bound blebbistatin still projects from the filament in a helical fashion. The biochemical studies on the effect of blebbistatin on rabbit fast skeletal myosin II and the structural studies on myosin II from *Dictyostelium discoideum* both suggest that blebbistatin stabilizes the so-called “closed state” (Kovacs, Toth et al. 2004; Allingham, Smith et al. 2005). This state has been associated with helical cross-bridge arrangement in muscle (Xu, Gu et al. 1999; Kovacs, Toth et al. 2004). The state of the myosin head with bound blebbistatin thus appears to be structurally similar to the dissociated myosin-ADP- P_i state rather than to the myosin-ATP state. Zhao and coworkers used negatively stained electron micrographs of thick filaments and reported very recently that the relaxed helical projections of the “closed” state were preserved in the presence of blebbistatin (Zhao, Padron et al. 2008). These results are consistent with our x-ray diffraction data from the organized contractile system of muscle that show the presence of layer lines similar to the relaxed state in the presence of blebbistatin.

4.5 MECHANISM OF BLEBBISTATIN INHIBITION

Kinetic studies on the myosin S1 fragments from rabbit fast skeletal muscle have shown that apoS1 binds blebbistatin weakly and that actin-bound S1 does not bind it at all (Kovacs, Toth et al. 2004). This would suggest that, when adenosine nucleotides are absent or when myosin heads interact with actin under the “strongly bound cross-bridge” configuration, myosin has no or little affinity for blebbistatin. To extend these observations from the isolated and purified muscle protein fragments in solution to those of the filaments constrained within a cellular lattice, we examined blebbistatin binding in rigor (paper II). Incubating permeabilized EDL with 10 μM blebbistatin for 20 mins under relaxed conditions, attenuated significantly the subsequent Ca^{2+} -induced

contraction. Adding blebbistatin under “rigor” conditions prevented this attenuation (fig.1, paper II). Thus, blebbistatin does not bind to the myosin when engaged in the “rigor cross-bridge” state, a significant extension of the previous biochemical analysis on isolated protein fragments.

The release of P_i is coupled to force generation in muscle (Goldman 1987), as illustrated in Figure 1 of the Introduction). Addition of P_i relaxes permeabilized muscle because the the P_i release reaction in Fig. 1 is forced in reverse. It is possible that blebbistatin inhibits force by affecting the rate constants and the equilibrium constant of the P_i release reaction. Therefore, we examined the effect of P_i on contraction in permeabilized pig ventricular muscle. Here, increasing P_i inhibited active force in a manner similar to that described previously for skeletal muscle (Tesi, Colomo et al. 2002). The P_i -force relationship was not altered by 10 μ M blebbistatin although the initial force with the presence of blebbistatin was lower (fig. 5, paper II). The results are consistent with a model in which blebbistatin does not bind to the AM states or interfere with the phosphate release reaction directly. Rather, blebbistatin inhibits force generation by trapping the cross-bridge in the state prior to P_i -release (M·ADP· P_i -blebbistatin). This state corresponds to the closed, detached state as detailed above.

The magnitude of the maximal shortening velocity of muscle (V_{max}) is thought to be limited by cross-bridge detachment rates and the ADP off-rate (Siemankowski, Wiseman et al. 1985). Since blebbistatin inhibits force in muscle by trapping dissociated cross-bridges, as discussed above, it seems unlikely that blebbistatin would influence the maximal shortening velocity. However, to exclude this possibility, we examined the effect of blebbistatin on V_{max} in permeabilized preparations from mouse cardiac ventricular muscle. For a comparison with the possible blebbistatin effect on V_{max} and force, we performed experiments at high ADP/low ATP conditions. In the high ADP/low ATP situation, the isometric force was inhibited by 30 % and V_{max} by about 40 % of the values in the absence of blebbistatin. This reflects the reduction of filament sliding by slowing of the cross-bridge detachment reactions. The lower force is most likely due to trapping of cross-bridges in AM and AM·ADP states which reduces the number of cross-bridges entering into the force generating cycle. When these experiments were repeated at 1 μ M blebbistatin, a different picture emerged: although force still was inhibited by 30%, only negligible effects were observed on V_{max} (inhibition by about 3 %). This suggests that blebbistatin does not interfere with the ADP release or with ATP induced detachment of myosin from actin. In addition, a cross-bridge from a myosin molecule which has blebbistatin bound, does not constitute an internal load to shortening. Although blebbistatin binding does not affect shortening velocity of smooth and cardiac muscle (Rhee, Ogut et al. 2006; Eddinger, Meer et al. 2007) and paper I), a correlation seems to exist between the IC_{50} and the V_{max} of different muscles (Section 4.3). This may suggest that the blebbistatin binding site on the myosin head can have a role in determining ADP off-rate and muscle shortening, i.e. that the structure of this region influence both filament sliding velocity and blebbistatin binding affinity.

Blebbistatin is light sensitive in the blue wavelength range (Kovacs, Toth et al. 2004; Ramamurthy, Yengo et al. 2004; Sakamoto, Limouze et al. 2005) and can be destroyed

by light. This was generally considered in our experiments (papers I and II) by using red light filters and dimmed light conditions. However, we also attempted to exploit this property of blebbistatin by studying the rate of force redevelopment from a starting point of blebbistatin-inhibited contractions through targeted photolysis of blebbistatin by sudden, instant, high energy blue light illumination. In permeabilized EDL and soleus muscles, we expected that a sufficiently fast destruction and removal of blebbistatin, could allow us to measure the rate of force generating cross-bridges. The illumination with a strong light flash resulted in some force redevelopment (fig. 3, paper II). The amplitude of the force transient was comparatively low, about 20% of maximal tension in the absence of blebbistatin. The light-induced contractions could be repeated. However, after 3-5 flashes, the final tension reached about 50% of the maximal tension. The rate constants for force redevelopment were about 1 s^{-1} and similar in the fast and slow muscle types (fig. 3, paper II). These rates are significantly slower than that reported on striated muscle following photolysis of caged-ATP in the presence of Ca^{2+} (Goldman, Hibberd et al. 1984). It has been shown that photodestruction of blebbistatin can generate toxic radicals or induce photochemical effects on proteins (Kolega 2004; Sakamoto, Limouze et al. 2005). This might contribute to the slow rate but we have not examined this possibility. In addition, the similar rates obtained in fast and slow skeletal muscle is suggestive of a rate-limiting step not directly associated with the intrinsic kinetics of the myosin molecule: release rates of photolyzed component or cross-bridge isomerization are some of the possible mechanisms that could be involved. Our results thus show that the inhibition by blebbistatin in muscle fibers can be partially reversed by photolysis of blebbistatin. The subsequent force regeneration is however not rate-limited by the force generating cross-bridge transitions.

4.6 EFFECT OF PYRIMIDINES ON CARDIAC MUSCLE

In paper III, the effects of pyrimidines (UTP and UDP) in cardiac muscle were examined. This collaborative study involves (i) determination of pyrimidine release in plasma in human patients with cardiac ischemia, (ii) determination of P2Y receptor expression, and (iii) determination of the cellular effects of pyrimidines on isolated cardiomyocytes. My experimental contribution relates to *iii*.

Three groups of patients with chest pain were included in the study: (i) infarction patients with elevated ST segments in electrocardiography (ECG) recordings, (ii) infarction patients without elevated ST segments and (iii) patients with chest pain without signs of cardiac infarction. Plasma level of UTP was increased in the infarction patients with elevated ST segments (fig. 1, paper III). Since UTP is rapidly degraded to UDP (Harden, Lazarowski et al. 1997), the plasma level of UTP might be much lower than the actual concentration at the cell surface. Possible physiological effects could thus reflect action of both UTP and UDP. It was furthermore observed that the plasma levels of ATP and UTP were correlated. A possible co-release of these nucleotides during ischemia might thus be postulated. In a previous study on pigs with cardiac ischemia, UTP measured from the coronary sinus was found increased (Erlinge, Harnek et al. 2005). Therefore, it is most likely that UTP can be released from the cardiomyocyte, although endothelial and blood cells may also contribute. These studies thus suggest that UTP and UDP are released from the ischemic myocardium in man.

UTP acts on both P2Y₂- and P2Y₄-receptors (Nicholas, Watt et al. 1996) whereas UDP acts on P2Y₆-receptors (Communi, Parmentier et al. 1996). A logical next step was thus the investigation of the expression of P2Y-receptors in human and mouse heart samples. mRNA-expression levels in the mouse heart was the highest for P2Y₂ and P2Y₄ whereas the latter was absent in the human heart. Very much lower expression levels were detected for P2Y₆ in both species (fig. 4 and 5, paper III). It should be noted, however, that expression levels of mRNA are not always tightly correlated with the magnitude of receptor mediated biochemical or physiological effects. For instance, in vascular smooth muscle it was shown that in vascular smooth muscles, P2Y₆ is more potently activated than P2Y₂ although their expression levels are similar (Malmsjö, Hou et al. 2000; Wang, Karlsson et al. 2002). P2Y₆ was also detected in the human heart using Western blot and immunohistochemistry (fig. 6, paper III). These results thus show that the receptors reacting to UTP and UDP are present in the myocardium.

To examine the effects of UTP and UDP on the shortening of isolated mouse cardiomyocytes, we used stable analogues to avoid possible transient effects due to metabolism. Thus we used UTP γ S to selectively stimulate P2Y_{2/4} and UDP β S for selectively stimulate P2Y₆ (Goody, Eckstein et al. 1972). Both compounds had positive inotropic effects, i.e. they increased the extent of cellular shortening (fig. 2, paper III). UTP γ S increased shortening by 52 %, an extent similar to that obtained by β_1 -adrenergic stimulation through isoproterenol (65%), suggesting that UTP has positive inotropic effects via P2Y₂ and/or P2Y₄ receptors. UDP β S also increased cardiomyocyte shortening (by 37%) an effect abolished by the P2Y₆-antagonist MRS2578, suggesting that the positive inotropic effect of UDP is mediated by the P2Y₆ receptor.

The mechanisms of the inotropic effect of UTP in the heart have been previously shown to be mediated via a phospholipase C (PLC) pathway (Podrasky, Xu et al. 1997). In addition, UTP has also been reported to increase cAMP in the frog heart (Flitney and Singh 1980). In study III, the inotropic effects of UDP β S and UTP γ S were inhibited by U73122, a selective PLC-inhibitor, but not by SQ22563 (a selective adenylyl cyclase inhibitor). These data thus suggest that the effects of UTP and UDP are mediated by a G_q-coupled receptor via the IP₃-signaling pathway and that the effects do not involve generation of cAMP.

In conclusion, paper III shows that the pyrimidines UTP (and possibly UDP) are released under ischemic conditions, have positive inotropic effects in myocardial cells mediated through P2Y-receptors and the PLC/IP₃ pathway. The clinical implication of pharmacological intervention with these receptors remains to be examined. As suggested in a recent review (Erlinge and Burnstock 2008), pyrimidine agonists may have a role in treatment of some conditions characterized with impaired cardiac contractility. Furthermore, pyrimidine antagonists could be useful in treating hypertension and congestive heart failure.

4.7 STRUCTURE AND FUNCTION OF SKELETAL MUSCLE OF ZEBRAFISH LARVAE

As stated in the Introduction, the zebrafish has become an important model in developmental biology and in studies on specific gene function (Langenau and Zon 2005; McGonnell and Fowkes 2006; Guyon, Steffen et al. 2007). In this aspect, the larva stage is of interest since the gene expression can be manipulated by injection of morpholino antisense approach. Previous work on muscle in zebrafish larvae has been focused on morphological and developmental aspects (Raeker, Su et al. 2006; Hinitz and Hughes 2007; Wohlgemuth, Crawford et al. 2007). Functional studies are very few and mainly restricted to movement analysis of whole larva (McDermid and Drapeau 2006). The zebrafish model can be used to examine how specific genes and proteins, of unknown function and/or clinical significance, influence muscle. Some of the examined genes/proteins might have large structural effects but functional studies are needed to understand subtle and perhaps (patho)physiologically more important changes in muscle. The size of the larva poses a technical challenge. We therefore attempted to develop techniques for studies of larval muscle function and structure. Our approach was to combine mechanical measurements using “classical” muscle physiology techniques with x-ray diffraction to examine muscle filament structure (paper IV, see also commentary (Squire, Knupp et al. 2008)).

A key issue for the mechanical and structural studies is the understanding of the muscle fiber orientation and filament arrangement. A previous study has shown that the muscle fibers of the larva are composed of fast and slow fibers. The superficial fibers are mainly in parallel with the zebrafish larva long axis whereas the deeper fibers are more helically arranged (van Raamsdonk, van't Veer et al. 1982). In the adult fish, an angular or helical arrangement of muscle is considered to be physiologically important since this structural arrangement enables the fast and slow fibers to work at different positions of their length tension relationships during tail bending and swimming (Rome and Sosnicki 1991). We included x-ray diffraction experiments on permeabilized adult zebrafish muscles and found that the muscle filaments, as judged with equatorial patterns, were oriented in two main directions (fig. 6, paper IV) with approximate 25° angle to the long axis. This is consistent with an angular arrangement of the muscles in the adult fish. We examined the arrangement of cells and filaments in the larval muscle. In contrast to the adult fish, the contractile filaments were oriented in parallel with the long axis in the larva, as shown by the images of muscle cells and the equatorial x-ray diffraction patterns (fig. 6, Paper IV and Figure 7 below). We cannot exclude a small angular distribution of filaments and cells, but the mean direction is clearly in parallel with the larvae.



Figure 7. Light microscopy picture of larva muscle in a preparation mounted for mechanical experiments as described in Methods. Note that the connective tissue attachments of the cells, the myosepta, are angled about 45°, but that the cells are in parallel with the larvae long axis. Arrow indicates a myoseptum. The bar is 20 μm .

The equatorial x-ray patterns of a single larval muscle preparation were strong and the 1.1 and 1.0 equatorial peaks could be clearly resolved (fig. 4, paper IV). We also determined the lattice spacing and the sarcomere lattice volume. Both sarcomere length and solution osmolarity influence the filament lateral spacing. We therefore performed the experiments at different sarcomere lengths and solution osmolarities and observed maximal active force in the physiological salt solution. Therefore we focused on this solution also in the structural study. At a sarcomere length of 1.9 μm and in a solution of 306 mosm/l, the average lattice spacing was 22.5 nm for the 1.1 reflection and 39.3 nm for 1.0 reflection (cf. Figure 3 in Materials and Methods). We calculated the lattice volume to $3.42 \times 10^{-3} \mu\text{m}^3$. The spacing values increased (i.e. the filament lattice swelled) when the osmolarity of the solution decreased and changed when the muscles were shortened or stretched. This shows that the external osmolarity and degree of stretch affect the muscle structure. Compared with mammalian muscles, zebrafish has a smaller lattice spacing and lattice volume. This is similar to those of frog (Millman 1998). This suggests a more dense packing of myofilaments compared with the mammalian muscles. In one set of experiments, we also measured the intensity of the equatorial reflections during active contractions. To get adequate resolution we added recordings during two active contractions (2 x 1 s tetanus). The intensity of 1.0 decreased whereas the intensity of 1.1 remained unchanged and the 1.1/1.0 ratio increased significantly compared with that in relaxed preparations. The changes of the intensities reflect mass transfer between thick and thin filaments during active contraction.

As discussed above, the sarcomeres were oriented mainly in parallel with the preparation long axis. Using light microscopy in combination with force measurements, we could determine the relation between force and sarcomere length. The force was dependent on degree of stretch and the optimal sarcomere length for active force development was 2.15 μm , which is similar to that reported for frog muscles (Altringham and Bottinelli 1985) and shorter than that of mammalian fast twitch muscles (Edman 2005). Previous studies of perch fish muscle (Granzier, Akster et al. 1991) have found different optimal length for fast twitch (1.9-2.2 μm) and slow twitch fibers (2.6-2.8 μm). The zebrafish larvae muscles thus appear to have an optimal sarcomere length similar to that for fast twitch fibers of adult fish.

Our preparation included the whole larva since the main part of the preparation cross-section is muscle and we also confirmed that the force responses were not affected if we removed the swim bladder, yolk sack and gut parts of the larva. The maximal active single twitch force developed at optimal length was about 0.5 mN. We estimated the cross-sectional area of the muscle in the preparation using published information on the shape of the muscle cross-section (van Raamsdonk, van't Veer et al. 1982) and obtained an approximate value of 35-50 mN/mm^2 for the single twitch active stress (i.e. active force per area). This is lower than values reported for single twitches and tetanic force of skeletal muscle of mouse about 100 mN/mm^2 and 370 mN/mm^2 , respectively (Crow and Kushmerick 1983; Edman 2005). At present we cannot clarify the reason for the comparatively low active force per cross-sectional area. Several possibilities may exist. The estimation of the relative amount of muscle in the cross-section may include some uncertainty and it is possible that the mechanical coupling of the cells influence force. The contractile protein content might be lower in the larvae. The force generation might

also be low due to a short duty cycle (i.e. the relative time a cross-bridge spends in attached state) associated with the fast contractile phenotype of the larvae (see discussion below). Further work is required to determine the force per cell cross-section in the zebrafish larvae muscle.

The single twitch contractions of the zebrafish larvae muscle were very rapid (fig. 2, paper IV), with half-times for contraction and relaxation of 3.2 and 4.7 ms, respectively. When the stimulus frequency was increased, summation and tetani were observed (fig. 3, paper IV). However the relation between force and stimulation frequency showed that summation effects were not prominent; the single twitch/tetanus force ratio was about 0.8. This is significantly lower than that in EDL muscle of mouse where the single twitch is about 28% of the tetanus (Sabbadini and Baskin 1976). In the zebrafish larva, a smooth tetanus was observed at a higher frequency (~190 Hz), compared with about 60~80 Hz in fast twitch muscles of mouse (Edman 2005). Figure 8 illustrates the force-frequency relationship of the larvae muscles. The fusion frequency is thus higher than that of mammalian and frog muscle and was close to that reported for swim bladder muscle 250~300 Hz (Rome, Syme et al. 1996). The data thus suggest that the zebrafish larval muscle expresses mainly a fast muscle phenotype.

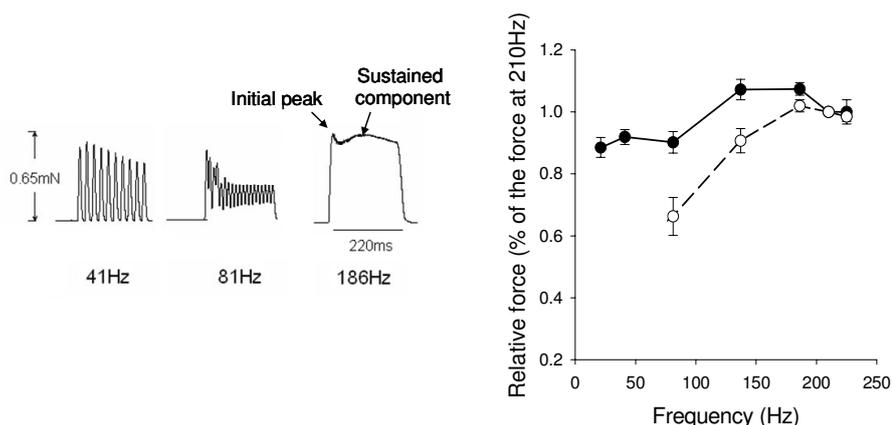


Figure 8. Left panel shows contractions at different stimulation frequencies of zebrafish larvae (220 ms tetanic stimulations). We examined the initial peak and the force at the sustained component (arrows). The right panel shows the force of the initial peak and of the sustained component at different stimulation frequencies. Illustration modified from fig. 3 in paper IV.

We observed that the force response at higher stimulation frequencies had an initial peak and a second more sustained component (Figure 8 above and fig. 3, paper IV). In the adult zebrafish, the fast muscle fibers are mainly used in escape response and occupy deep portions of the body. The slow muscle fibers are mainly utilized in sustained swimming and are located in the wedge shaped region between skin and the superficial cells (van Raamsdonk, van't Veer et al. 1982). Although the contractile responses (i.e. the fast single twitches, high summation frequencies, high fatigue sensitivity) of the larval muscle suggest that the main force is generated by a comparatively fast muscle phenotype, it is possible that slower fibers, which are present

in the muscle (Devoto, Melancon et al. 1996; Hinits and Hughes 2007) contribute during later phases of the tetani. Even if the amount of slow twitch fibers is low (Devoto, Melancon et al. 1996), they might be able to generate more force when stimulated at higher frequencies and contribute to tension when the bulk of the faster fibers fatigue and generate less force. To examine the possibility that two contractile components, fast and slow, contribute to contraction in the larva muscle, we used BTS which is suggested to attenuate active force of fast twitch muscle and to be less effective in slow skeletal muscle (Cheung, Dantzig et al. 2002). We found that BTS suppress both the initial and sustained components of the tetanus (fig. 3, paper IV). The attenuation of the sustained component by BTS was less, compared with the effect on the initial phase, which is consistent with a model where a small component of slower fibers contributes to sustained contraction in the larva.

A fast contractile phenotype of the muscle can also be reflected in its fatigue sensitivity. This is obvious in the living larvae which swim with short bursts of activity. To examine the fatigue properties in the isolated muscle preparations, we used a fatigue stimulation protocol with repeated short tetani (Westerblad, Allen et al. 1998). We chose 220 ms tetanus duration and a frequency giving smooth tetanic responses (186 Hz) and increased the train rate of the tetanic stimulations. The tetanic force significantly decreased at a low train rate (force decrease to 50% at a tetanus train rate of 0.1 s^{-1}). This decrease is more pronounced than that observed in mammalian fibers (Westerblad, Allen et al. 1998; Balogh, Li et al. 2003) and suggests a high fatigue sensitivity of zebrafish larvae muscle. The reason for the decrease in force is complex and has been shown in mammalian fibers to be related to a reduced Ca^{2+} release (Edman and Lou 1992; Westerblad, Allen et al. 1998). The zebrafish larva has a fast muscle phenotype, which most likely requires rapid Ca^{2+} -release and uptake. The fatiguing contractions can put a significant strain on Ca^{2+} handling mechanisms and cell metabolism. At present, we do not know the properties of the muscle cell metabolism in the larvae and it is possible that the metabolic systems have a reduced capacity to handle generation of ATP or removal of products during sustained contractile activity.

We adapted a skinning procedure to the zebrafish larvae preparations (paper IV). In general, the force was lower after permeabilization (~20 % of single twitch force). We activated the preparations at different free $[\text{Ca}^{2+}]$ and compared the Ca^{2+} -sensitivity with that of permeabilized fast mammalian muscles (mouse EDL preparations). The Ca^{2+} -sensitivity of the zebrafish larvae was significantly lower (fig. 5, paper IV). This is consistent with a low Ca^{2+} -affinity of the thin filament regulatory system and a change in the binding or dissociation rates for Ca^{2+} to troponin C. It seems unlikely that the rate of Ca^{2+} -binding is slow. Therefore one possibility is that the low affinity is the result of a fast Ca^{2+} -dissociation rate. A rapid Ca^{2+} -dissociation rate from troponin might be physiologically important in the fast contractions of the zebrafish muscle.

In conclusion, our study of the zebrafish larvae muscles show that mechanical measurements with high resolution can be performed on these muscles. A major advantage is the orientation of fibers and filaments which makes the extrapolation from preparation mechanics to sarcomeres, filaments and cross-bridges more straight forward. The contractions exhibited many characteristics of a fast muscle phenotype, but may also contain a small contribution from slower muscle fibers, as suggested by

the BTS experiments. The structural signals were very strong and several key structural features of the sarcomere, e.g. lattice spacing and mass transfer, could be recorded. Future work has to explore the meridional patterns and possible layerlines. The mechanical and structural approach based on the equatorial patterns in combination with the morpholino knock-down approach enable novel experiments determining the function of key cytoskeletal structures (e.g. desmin and C-protein).

5 CONCLUSIONS

- Blebbistatin is a strong inhibitor of actin-myosin interaction in the organized contractile system of striated muscle.
- Blebbistatin traps striated muscle myosin in a 'closed' state dissociated from actin.
- The inhibitory action of blebbistatin is dependent on muscle type, with a stronger effect in faster muscle.
- The inhibitory action of blebbistatin in cardiac muscle is not due to effects on the Ca^{2+} influx or activation systems.
- Both UTP and UDP induce positive inotropic effects on mouse cardiac myocytes via PLC-mediated signaling, independently of cAMP.
- Skeletal muscles of zebrafish larvae can be used for structural and functional studies.
- The cells and contractile filaments of skeletal muscles in the zebrafish larva are mainly in parallel with larval long axis.
- The zebrafish larval muscles exhibit a fast contractile phenotype with high fatigue sensitivity, high summation frequency and low Ca^{2+} -sensitivity.

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