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Low chloride conductance myotonia - in vitro investigations on muscle stiffness and the warm-up phenomenon

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ABBREVIATIONS

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ADR	arrested development of righting response
AP	action potential
ATP	adenosine tri-phosphate
9-AC	Anthracene-9-carboxylic-acid
BK	big conductance calcium activated potassium channel
$[Ca^{+2}]_i$	intracellular calcium concentration
$[Cl^-]_o$	extracellular chloride concentration
ClC-1	mammalian skeletal muscle chloride channel monomer
CLCN1	gene encoding skeletal chloride channel type 1
DHPR	dihydropyridine receptor
DM	myotonic dystrophy
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
EMG	electromyography
EMHG	european malignant hyperthermia group
ENU	ethylnitrosourea
ETn	early transposon
gCl^-	chloride conductance
HyperPP	hyperkalemic periodic paralysis
IVCT	in vitro contraction test
$[K^+]_o$	extracellular potassium concentration
K_{ATP}	ATP-sensitive potassium channel
KCNQ	human voltage-gated potassium channel
Kir2.1	inward rectifier potassium channel
K_v	voltage gated potassium channel
MH	malignant hyperthermia
Na^+/K^+-ATPase	sodium potassium pump
$Na_v1.4$	skeletal muscle sodium channel alpha subunit
NKCC1	sodium potassium chloride cotransporter type 1
PAM	potassium-aggravated myotonia
PC	paramyotonia congenita
PCR	polymerase chain reaction
RyR1	ryanodine receptor type 1
SCN4A	gene encoding skeletal muscle sodium channel alpha subunit
SERCA	sarcoendoplasmic reticulum Ca^{+2} release
SJS	Schwartz Jampel syndrome
SR	sarcoplasmic reticulum
T-system	transverse tubular system
WT	wild-type

1 INTRODUCTION

1.1 MYOTONIA - HYPEREXCITABILITY OF SKELETAL MUSCLE

Myotonia is the clinical description of a transient involuntary contraction of skeletal muscle experienced as muscle stiffness (Fig. 1). By definition, it is an electrophysiological dysfunction of the muscle fibers themselves. On electromyographic (EMG) examination, myotonic muscles exhibit myotonic runs, high frequency membrane discharges after trains of voluntarily evoked action potentials. The characteristic pattern reminds of a "dive-bomber" sound. In mild cases, myotonia may not be evident on clinical examination, yet the EMG may reveal the typical myotonic burst. The uncontrolled activity accounts for the "aftercontractions" which are the basis of the muscle stiffness. The hereditary myotonic syndromes are grouped according to their pathogenesis and clinical features (see Table 1). The most common forms of myotonia are dominant and recessive myotonia congenita (Rüdel et al. 1994, Lehmann-Horn et al. 2007).

1.2 LOW CHLORIDE CONDUCTANCE MYOTONIA

1.2.1 Thomsen's and Becker's myotonia

Myotonia congenita was first described in the late 1870s by the Danish physician Julius Thomsen, who himself suffered from the disease. Later, Peter Emil Becker convincingly proved the existence of recessive myotonia congenita characterized by later onset, moderate to severe myotonia with transient weakness. Thus, myotonia congenita was divided into a recessive disorder (Becker's myotonia), and dominant disorder (Thomsen's myotonia). The clinical syndrome is characterized by a relaxation deficit after forceful voluntary contractions. The inability of skeletal muscle to relax accounts for muscle stiffness. Its development is most prominent when a muscle has been rested for more than 10 minutes and is then strenuously activated for a few seconds. The then occurring involuntary "after-activity" may slow muscle relaxation by several seconds. In such a case "transient weakness" may accompany the myotonia (Rüdel et al. 1994, Lehmann-Horn et al. 2007).

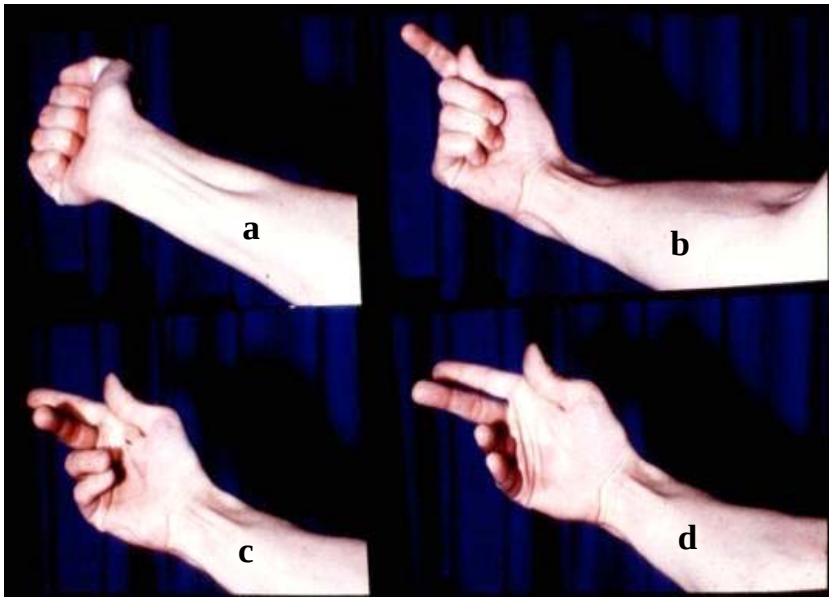


Figure 1: Myotonic stiffness in a patient suffering from Becker's myotonia.

After a forceful closure (a), the patient is unable to open the fingers fully (b). It takes the patient several seconds to reopen the fist (c). After one minute, finger 4 and 5 are still flected (d).

Becker's myotonia is more severe than Thomsen's myotonia and progresses slowly during childhood and adolescence. Patients are especially disabled when gross muscle is affected. Thus, patients with Becker's myotonia are more handicapped in daily life. Myotonic stiffness becomes obvious when the patient makes a tight fist after a period of rest (Fig. 1): the force exerted by finger flexors vanishes almost completely within a few seconds (Deymeer et al. 1998, Lehmann-Horn et al. 2007).

Neither Becker's nor Thomsen's myotonia presents with muscular dystrophy. In some patients slight myopathic changes with increased occurrence of central nuclei and pathologic variation of fiber diameter may be found. Hypertrophy of oxidative fibers, especially of type IIa fibers and a reduction in number or a complete absence of type IIb fibers is a common feature or is often seen (Crew et al. 1976, Jurkat-Rott et al. 2002, Wu and Olson 2002).

Both, Thomsen's and Becker's myotonia, are linked to defects in the gene encoding chloride channel type 1 (CLCN1) in skeletal muscle (Fig. 2). Both forms are referred to as low chloride conductance (gCl^-) myotonia, because gCl^- is markedly reduced (Kwiecinski et al. 1988).

1.2.2 Pathophysiological background

The muscle membrane has a high gCl^- (~ 80% of the total resting membrane conductance) (Steinmeyer et al. 1991, Chen et al. 1997). gCl^- stabilizes the resting membrane potential at the value predicted by the Nernst equation for chloride (about –80 mV). A decrease of gCl^- decreases the electrical stability, i.e. it causes membrane hyperexcitability. An experimental gCl^- decrease gCl^- to 20% is associated with a clear myotonic muscle behavior whereas reduction of gCl^- to 50% does not cause myotonia. These findings are substantiated by the fact that heterozygous carriers of recessive mutations are clinically asymptomatic. Some show mild EMG myotonia. The tacit assumption of 1:1 allelic expression in such cases may not be true (Barchi 1978, Kwiecinski et al. 1988, Chen et al. 1997).

gCl^- is crucial for countering the depolarizing effect of K^+ accumulation in the transverse tubular system (T-system) and for volume control of the T-tubule (Palade and Barchi 1977, Gosmanov et al. 2003, Kristensen et al. 2006). The T-tubule is characterized by a long (~2.4 µm length) narrow shape and a tight opening (~10-50 nm diameter) on the sarcoplasmic face. It has a regular arrangement along the myotubes and forms in conjunction with the sarcoplasmic reticulum (SR) triads inside the cells. These anatomic properties hinder diffusion between inner and the outer extracellular space and K^+ concentration ($[K^+]$) can rise significantly in the T-system during muscle activation (Dulhunty 1984, Flucher et al. 1993, Chawla et al. 2001).

The action potential (AP) generation and repolarization cause K^+ efflux. The efflux of K^+ associated with a single AP increases its tubular concentration by 0.4 mM (Kirsch et al. 1977). Activation of gross muscle during exercise leads to a rise in serum K^+ levels of more than 5 mM and the interstitial K^+ can reach values as high as 10 mM during fatiguing exercise. K^+-accumulation in the T-tubular lumen depolarizes the membrane sufficiently to initiate self-sustaining AP causing a prolonged (myotonic) contraction (Adrian and Bryant 1974, Juel et al. 2000, Pedersen et al. 2005).

1.2.3 Warm-up phenomenon

The warm-up phenomenon is a conspicuous and use-dependent feature of low gCl^- myotonia. With continued activity or repeated contractions of a muscle the myotonic stiffness is reduced or abolished. Also, the phenomenon of transient weakness disappears. The warm-up effect is short-lived, wearing off after rest for approximately 5-8 minutes but

some authors state that exercise as such may have beneficial long-term effects (Birnberger et al. 1975, Colding-Jorgensen 2005).

The warm-up phenomenon is not confined to low gCl^- myotonia (Table 1). It also occurs in other forms of myotonia which are Na^+ channel ($Na_v1.4$) defects and defects of cytoskeletal proteins e.g. Schwartz Jampel syndrome (SJS).

The mechanisms of the warm-up phenomenon remain unexplained. However, it is known that physiological as well as environmental factors influence the warm-up phenomenon.

Table 1: Factors influencing the various myotonic syndromes.

Channel defect	Cl^- channel (CLCN1)			Na^+ channel (SCN4A)			Other cause
Clinical presentation	Myotonia congenita		DM	HyperPP	PC	PAM	SJS
	Thomsen (dominant)	Becker (recessive)	Myotonic dystrophy	Hyperkalemic periodic paralysis	Paramyotonia congenita	Potassium aggravated myotonia	Schwartz jampel syndrome
Warm-up phenomenon	✓	✓	✓	↔	Paradoxical myotonia	↔	✓
Exercise	↓	↓	↓	(↑)	↑	↓↑	?
K^+-effects	?	?	?	↑	↑	↑	?
Cold	?	?	↔	↑	↑	↔	↔
Water deprivation/ Carbohydrate-rich meals	↓	↓	?	↓	↓	?	?

1.3 PHYSIOLOGICAL AND MICROENVIRONMENTAL MODIFYING FACTORS

Many patients report short- and long-term variability of their symptoms. Microenvironmental conditions, local build-up of metabolites etc, as shown in Table 1 influence the myotonic syndrome. Strenuous exercise goes along with production of lactic acid. This causes a local build-up in extracellular potassium concentration ($[K^+]_o$), a

decrease in tissue pH-level and an increase of extracellular osmolarity due to accumulation of metabolites (Juel et al. 2000, Pedersen et al. 2005, Hess et al. 2005).

Systematic studies of the influence of environmental factors have been carried out only with myotonic animals. Bryant et al. (1968) observed myotonia over a period of 7 months in a colony of eight myotonic goats. No correlation was found with variations in changes of indoor temperature, humidity, or atmospheric pressure. In contrast, $[K^+]$ in serum of myotonic goat (4.98 mM) was significant higher than in control (4.45 mM). In resting conditions serum osmolarity did not differ from control animals. However, myotonic stiffness was abolished in water deprived animals and recurred when the water deprivation was discontinued (Hegyeli and Szent-Gyoergyi 1961, Bryant et al. 1968). Moreover, carbohydrate-rich meals or intake of glucose can prevent or alleviate myotonic crises. Hunger aggravates the symptoms in low gCl^-myotonia patients as well as in Na^+ channel myotonia. Na^+ channel myotonia is characterized by an exacerbation of muscle stiffness by exercise, and cold environment.

Some patients claim that emotional factors, physical fatigue, psychological stress inconsistently aggravate myotonia. In Becker's patients, the severity of myotonia may be somewhat more pronounced in men than in women. Women often report that pregnancy worsens myotonia. Hypothyroidism also aggravates both muscle stiffness and weakness. Of the many orally administered drugs tested, mexiletine, a Na^+ channel blocker, is the drug of choice, however its therapeutic index is narrow (Colding-Jorgensen 2005, Lehmann-Horn et al. 2007).

1.4 GENETICS

The causative genetic defect for low gCl^- myotonia is found in CLCN1 on chromosome 7q encoding the voltage-gated Cl^- channel of the skeletal muscle fiber membrane (Koch et al. 1992, George et al. 1993). The chloride channel protein, ClC-1, is a homodimeric complex having two ion-conducting pores ("double-barrel"; Saviane et al. 1999, Fahlke 2001, Mindell et al. 2001, Dutzler et al. 2002). More than 70 mutations have been identified in CLCN1 (Fig. 2, Lehmann-Horn et al. 2007). CLCN1 is strongly expressed in skeletal muscle, although low transcript levels are also found in kidney, heart and smooth muscle (Koch et al. 1992). Although nonsense and splicing mutations usually lead to the recessive phenotype, missense mutations occur in both Thomsen's and Becker myotonia. In the

premolecular era the prevalence of Thomsen's myotonia was overestimated. Now the prevalence is estimated to ~1:400,000. The prevalence of Becker's myotonia is estimated to 1:25,000 (Jurkat-Rott et al. 2002).

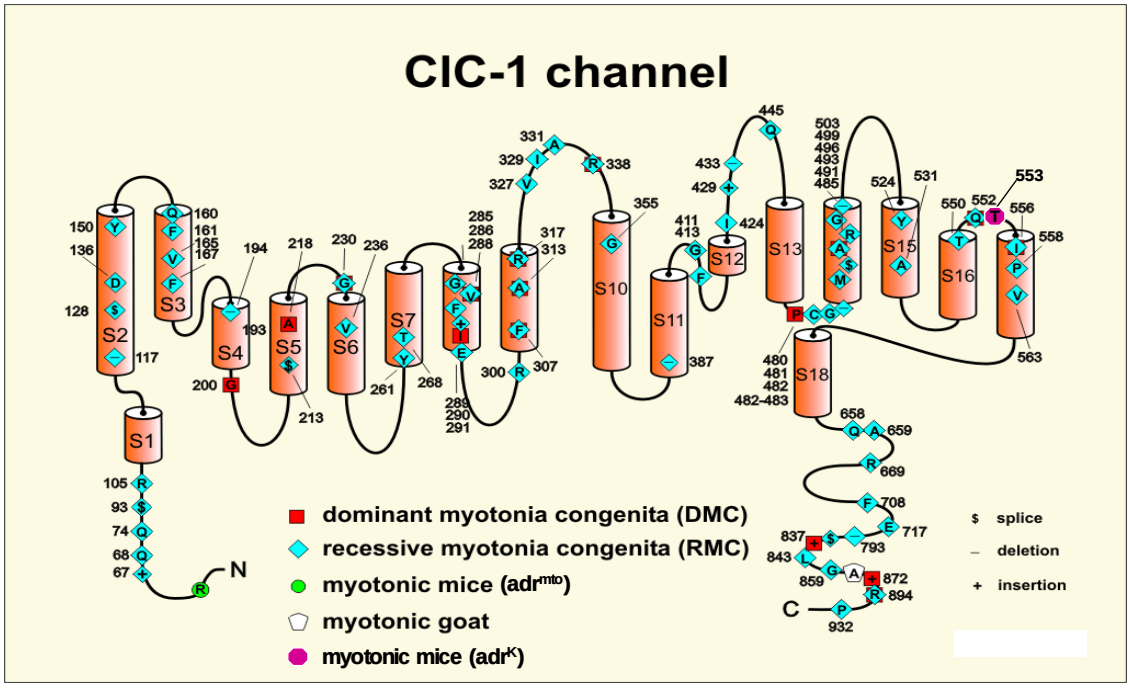


Figure 2: Membrane topology of the chloride channel.

The model shows the skeletal muscle chloride channel monomer, ClC-1. The functional channel is a homodimer encoded by the CLCN1 gene. The different symbols used for the known mutations leading to either dominant or recessive myotonia in human, mouse (adr^{mto}- and adr^{K}- mice) and goat are indicated. Conventional one-letter abbreviations are used for replaced amino acids.

1.5 Different Expression Patterns of Sarcolemmal and T-Tubular Membrane Proteins

The T-tubular membrane is characterized by a high density of the major inward rectifier potassium channel (Kir2.1), which is almost undetectable in the sarcolemmal membrane. This channel usually contributes to a wide variety of physiological functions such as the modulation of cell excitability, repolarization of AP, and determination of the cellular resting potential. It protects the membrane against hyperpolarization, which usually occurs short after AP-repolarization (Wallinga et al. 1999, Kristensen et al. 2006).

The T-tubular membrane also shows a higher expression of the big conductance Ca^{+2} activated K^{+} channel (BK) compared to sarcolemmal membrane (Kristensen et al. 2006).

The BK channel is activated in hypertonicity, by membrane depolarization and by an increase of $[Ca^{2+}]_i$ (Jacquemend and Allad 1998, Clerck et al. 2005). On the sarcolemma, opening is induced by Ca^{2+} entering through nicotinic receptors (Mallouk and Allard 2002). The channels enhance the repolarization by restoring the K^+-gradient and are contributing to K^+ release during AP because they behave much like the voltage-gated potassium (K_v) channels (Kristensen et al. 2006).

Another K_v channel, enhancing repolarization of skeletal muscle membrane, is the voltage gated potassium channel KCNQ5. The KCNQ5 belongs to the KCNQ family which so far consists of five members, KCNQ1-5. It can form a functionally heteromeric channel with the KCNQ3 alpha subunit, which is important for trafficking (Cooper et al. 2000, Gutman et al. 2003). The KCNQ3/5 yields currents that activate slowly with depolarization and are pharmacologically blocked by Linopirdine and 10, 10-bis (4-Pyridinylmethyl)-9(10H)-Anthracenon (XE 991) (Lerche et al. 2000, Schwarz et al. 2006).

A skeletal muscle K^+ channel, which is activated by depletion of intracellular ATP, is the ATP-sensitive K^+ channel (K_{ATP}). K_{ATP} channels are active at rest and contribute to the accumulation of interstitial K^+ because of their responsibility for K^+-release. However, they obviously do not play a major role in myotonic syndromes. The distribution of the K_{ATP} channels is similar to the sodium potassium transport system (Na^+/K^+-ATPase), i.e. is higher in the surface membrane than in the T-system (Nielsen et al. 2003, Kristensen et al. 2006).

The Na^+/K^+-ATPase extrudes three Na^+ ions in exchange for the uptake of two K^+ ions by using the energy from hydrolysis of one molecule of ATP. This system is necessary for the maintenance of the normal membrane potential and can contribute to stabilize the K^+-equilibrium (Clausen 1986). The α2-Na^+/K^+-ATPase, not α1-Na^+/K^+-ATPase has been shown to be present not only on sarcolemmal but also in the T-system of skeletal muscle fiber (Casademont et al. 1988, Horgan and Kuypers 1988, Cougnon et al. 2002). The density of Na^+/K^+-ATPase is lower in the tubular membranes than at the fiber surface at resting conditions (Fambrough et al. 1987, Kristensen et al. 2006).

A specific feature and relevant contribution to ion homoeostasis is the Na^+, K^+, $2Cl^-$ cotransporter type1 (NKCC1). NKCC1 is widely expressed including skeletal muscle, particularly in the sarcolemmal membrane, but also in the T-system although with less density (Delpire et al. 1994, Kristensen et al. 2006). It is involved in the maintenance of the fiber volume. NKCC1 is activated by hyperosmolarity resulting mainly from K^+ efflux and

lactate production during muscle contraction and from increased dietary intake. In resting skeletal muscle, it accounts for ~15% of K^+ uptake and ~23% of Na^+ uptake, and it has been shown to account for >30% of K^+ transport during muscle stimulation with either catecholamines or electrical stimulation (Lindinger et al. 2002).

1.6 PHARMACOLOGICALLY INDUCED LOW gCl^- MYOTONIA

The low gCl^- myotonic signs can be induced in vitro by the replacement of extracellular Cl^- ($[Cl^-]_o$) by the impermeant anion methane sulfonate. Bryant (1976) showed that normal goat fibers in the Cl^--free medium behaved remarkably like myotonic fibers. Not only was the membrane resistance equivalent to that of the myotonic fibers, suggesting a lack of gCl^-, but the fibers had also the typical myotonic features.

Anthracene-9-Carboxylic-Acid (9-AC) is a blocker of ClC-1. The myotonia-inducing effects have been confirmed in vitro as well as in vivo (Moffett and Tang 1968, Bryant et al. 1968). Barchi et al. showed that 50 μM 9-AC reduced gCl^- by >90%. The resting membrane potential (RMP) and AP amplitude were not significantly altered (Palade and Barchi 1977, Furman and Barchi 1978).

1.7 ANIMAL MODELS OF LOW gCl^- MYOTONIA

About 30 years after the first description of myotonia in humans, White and Plaskett (1904) described a breed of “fainting” goats raised in Tennessee. The animals have attacks of extreme muscle stiffness when attempting a quick forceful motion, so that they often fall to the ground for 5 to 20 s with extended neck and limbs. Clark et al. (1939) were the first to refer to the disease as “a form of congenital myotonia in goats”. The disorder is transmitted as an autosomal dominant trait. The susceptibility to malignant hyperthermia (MH) was excluded for those goats (Newberg et al. 1983).

In the late 1970s, two spontaneous mouse mutations were detected, one in the A2G strain discovered by Watts and Wattkins (1978, 1984) in London, UK, the other in the SWR/J strain, discovered by Heller et al. (1982) in Bar Harbor/Maine, USA. The behavioral abnormalities of the affected animals were very similar, and in either mutant the disorder was transmitted as an autosomal recessive trait. The British scientists were struck by the observation that from days 10 to 12 onwards the affected animals had difficulty in righting

themselves when placed supine and therefore called the mutation “ADR” for “arrested development of righting response” (Fig. 3).

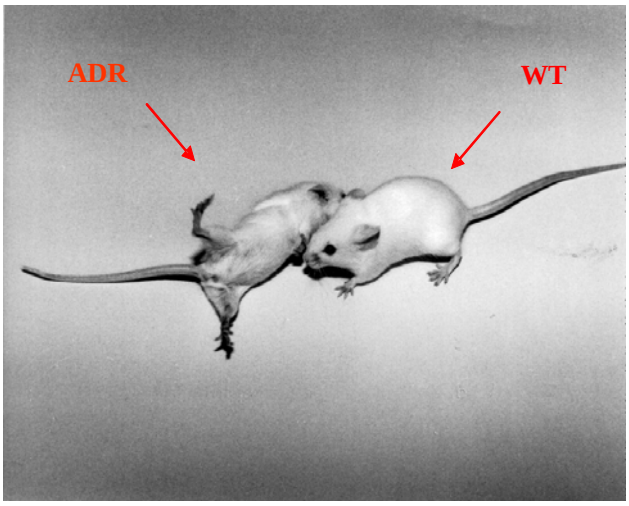


Figure 3: ADR and WT mouse.

The severe muscle stiffness in a myotonic ADR mouse (left) induced by muscle activity compared with a normal control mouse (WT, right). Both animals had been laid on back at same time and tried to right themselves.

The American researchers named the strain “MTO” for “myotonic” because electrical myotonia was recorded in the EMG from the stiff muscles. The allele has been named adr^{mto} (Heller et al. 1982, Jockusch et al. 1988). Later, an ethylnitrosourea (ENU)-induced mutation in the adr allelomorphic series, named adr^{K} has been reported (Neumann and Weber 1989). Every mouse mutant is due to a defect at one and the same gene CLCN1, which is located on the chromosome 6 (Davisson et al. 1989, Jockusch et al. 1990). The standard allele of adr mouse is due to the insertion of a retroposon of the early transposon (ETn) type to ClC-1 that destroys the gene’s coding potential for several membrane spanning domains (Fig. 2, Steinmeyer et al. 1991). The adr^{mto} allele is due to a stop codon (in place of Arginine; R) that truncates the ClC-1 polypeptide of N-terminal position 47. The ENU–induced allele adr^{K} is a missence mutation, changing an Isoleucine (I) to Threonine (T) amino acid at position 553 in exon 13 (Gronemeier et al. 1994). The defect of ClC-1 allele ($ClC\text{-}1^{adr}$ allele) causes a loss of 4.5 kb ClC-1 RNA, and mice heterozygous for the defective $ClC\text{-}1^{adr}$ contain about 50% functional mRNA in their muscle compared with wildtype mice. But the gene dosage is enough for sufficient ClC-1 expression and there is no significant difference in gCl^{-} compared to wild type mice (Chen et al. 1997).

1.8 AIMS OF THE STUDY

Mutations in the CLCN1 gene cause membrane hyperexcitability in skeletal muscle. The resulting transient muscle stiffness is characterized by aftercontractions and a slowed relaxation (i.e. myotonia). By unknown mechanisms, low gCl^- myotonia shows a reduction of stiffness after exercise. The so called warm-up phenomenon is poorly understood. However, it is known that physiological and environmental factors influence the myotonic syndrome. Therefore, the main goal of this study was to clarify the influence of these factors on myotonic stiffness, particularly, on the mechanisms of the warm-up phenomenon. The effects of pharmacological substances which influence ion conducting proteins (i.e. $Na_v1.4$, KCNQ5, BK, NKCC1) were studied and evaluated for potential clinical benefit.

This in vitro study investigates pharmacologically induced low gCl^- myotonia as well as muscle tissue of myotonic ADR mice. The functional and electrophysiological studies were conducted by mechanographic registrations and membrane potential measurements.

2 MATERIALS AND METHODS

2.1 MOLECULAR BIOLOGY

2.1.1 Animals and breeding

Myotonic ADR mice were used as an animal model for low gCl^- myotonia. The mutant A2G strains of heterozygous (adr/+) animals were originated from H. Jockusch, the University of Bielefeld, Germany (Jockusch et al. 1988). To obtain ADR (adr/adr) mice, Balb/c x adr/+ females were mated with Balb/c x adr/+ males in the central animal research unit of Ulm university and kept in the essential specific pathogen-free animal facility of the university. The adr allele was verified using polymerase chain reaction (PCR) analysis (see the following sections). The homozygous (adr/adr) offsprings that were distinguished from heterozygous mice (adr/+) by their myotonic phenotype manifest from day 7 onward were used as low gCl^- myotonic mice.

2.1.2 DNA extraction

The presence of the pathogenic mutations in the animals was tested using PCR analysis (Krämer et al. 1998). DNA was extracted from homogenized mouse tail tips (4-6 mm) and purified using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

4-6 mm of a mouse tail tip was homogenized with 180 µl of tissue lysis buffer (ATL buffer) and 20 µl proteinase K reagent in a 1.5 ml tube. The samples were gently mixed and then incubated at 56°C for 2 h. After the tissue was completely dissolved, the samples were briefly centrifuged, brought to volume with 200 µl lysis buffer (AL buffer), mixed and incubated at 70°C for 10 min, and then centrifuged at full speed for 30 s to remove drops from inside the lid.

200 µl of 100% ethanol was added to each sample. The homogenized solution thoroughly mixed before a brief centrifugation for removal of drops from inside the lid. The mixture was transferred to the QIAamp Spin columm (in a 2 ml collection tube) and centrifuged at 6,000 x *g* for 1 min. The tubes containing the filtrates were discarded while the QIAamp Spin Column was transferred to a 2 ml collection tube and it was continued with the DNA washing step.

500 µl of the first wash buffer (AW1) was added to each sample from DNA precipitation followed by centrifugation at 6000 x *g* for 1 min. Each QIAamp Spin Column was

transferred to another 2 ml collection tube and the flow-through was discarded. In the same procedure as above, DNA samples were washed again with 500 µl second wash buffer (AW2) and centrifuged at full speed (20,000 x g) for 3 min. They were then eluted from the QIAamp spin column by adding 200 µl of elution buffer, incubation at room temperature for 5 min and centrifugation at 6000 x g for 1 min. The elute was kept in a 1.5 ml collection tube and the column was discarded. The DNA in the tube was inspected, quantified and subjected to PCR. When not in use, DNA aliquots were stored at -20°C.
To determine the concentration of the DNA probes, the extinction A_{260} at 260 nm optical wavelength was measured on an automated spectrophotometer (Gene Quant™ II, Amersham Pharmacia Biotech, Freiburg, Germany). Appropriate nucleic acid dilution was prepared to obtain an extinction coefficient between 0 and 1. Optical density of 1 corresponds to a concentration of 50 µg/ml for DNA probes. The DNA concentration was calculated the following way applying Lambert-Beer's Law:

$$A_{260} \text{ x dilution factor x } 50 = \mu g/ml \text{ DNA}$$

A qualitative test was done by measuring the ratio A_{260}/A_{280} (extinction coefficient at 260 and 280 nm wavelength). A ratio between 1.6 and 2 was considered to indicate purified DNA.

2.1.3 Polymerase chain reaction (PCR)

PCR was used to amplify DNA fragments from genomic DNA. PCR amplification was performed using 40-80 ng of genomic DNA in a final volume of 50 µl containing a mixture of the following: 1 µl DNA (40-80 ng); 41.3 µl distilled water; 5 µl 10 x PCR buffer (15 mM $MgCl_2$; Qiagen, Hilden, Germany); 1 µl primer ADR-forward (50 pmol/µl; Interactiva, Ulm, Germany); 1 µl primer ADR-reverse (50 pmol/µl; Interactiva, Ulm, Germany); 0.5 µl dNTP-mixture (dGTP, dATP, dTTP, dCTP) (10 mM; Sigma-Aldrich, Steinheim, Germany); 0.2 µl Taq-DNA-polymerase = 1 U (Qiagen, Hilden, Germany)
For the detection of the adr allele, genomic DNA was amplified using (from 5´to 3´) CTG TCC AAC CTA AAC TCT CAA GC as forward primer and TCC TAC CGC ATC CTC AGC AA as reverse primer.
PCR amplification was done using a Techne Progene Thermocycler (Thermodux, Wertheim, Germany). It represents a cycle-repeating reaction, with each cycle including

three steps of denaturation, annealing and extension. The program was set for sequencing PCR reactions (Table 2).

Table 2: Conditions used for PCR reactions.

Number of cycles	Temperature	Duration
start	94°C	3 min
39	94°C	1 min
39	57°C	1 min
39	72°C	2 min
end	4°C	

2.1.4 DNA-agarose gel electrophoresis

Double stranded DNA fragments were separated by agarose gel electrophoresis. The gel concentration used was 1.8% agarose (Biozym, Oldendorf, Germany) and was heated (100°C for 2 min) in 1 molar 10 x TBE Buffer (Tris-Borate 45 mM; EDTA 1 mM, pH 8.3; Merck, Darmstadt, Germany). After cooling to 50-55°C, 8 µl of 0.7 µg/ml of ethidiumbromide (Eurobio, Lesulis, France) was added and a horizontal gel was prepared in a mini-chamber (Peqlab, Erlangen, Germany). DNA probes were loaded with 1/5 final volume 6 x loading buffer (15% Ficoll, 0.1% Bromphenolblau; Merck, Darmstadt, Germany). As a standard DNA marker, 10 ng from a 100 bp or a 1 kb marker (Eurogentec, Karlsruhe, Germany) was used. Conditions used for gel electrophoreses were 100 V, 50°C for 1 h. Nucleic acids were detected by UV illumination and the results were documented photographically.

The presence of the adr allele resulted in a PCR product of about 600 bp (calculated: 594 bp) both for homozygous (adr/adr) and heterozygous animals (adr/+), whereas no band was seen from the wild type (+/+) allele (Fig. 4).

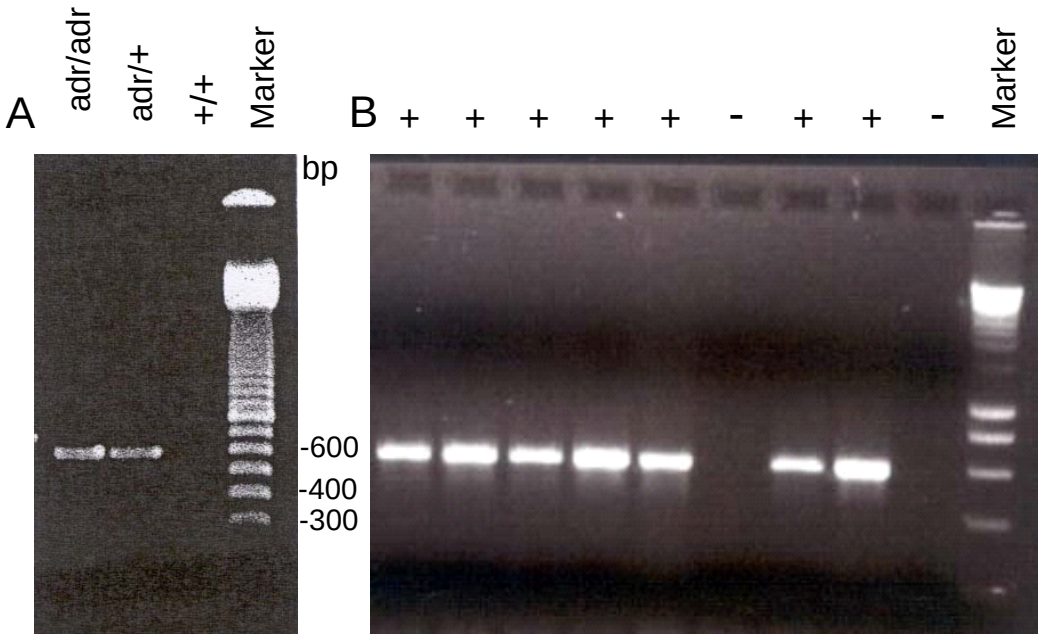


Figure 4: Analysis of adr alleles using the PCR.

A: PCR products amplified from DNA of myotonic (adr/adr) heterozygous (adr/+), and control (+/+) mice. In the presence of the allele, a PCR product of roughly bp appears (two lanes) whereas no such product is seen with the wild type allele only. B: The allele-specific products of adr alleles have characteristic lengths and are only visible if the corresponding mutation is present in the template. + positive; - negative

2.2 SOLUTIONS AND SUBSTANCES FOR FUNCTIONAL TESTING

2.2.1 Solutions

For the muscle contraction experiments and electrophysiological experiments physiological salt solutions were used with similar components as physiologically found in the extracellular environment. Approximately 15 min before the start of an experiment and during the whole experiment the solutions were always bubbled with carbogen (MTI IndustrieGASE, Neu-Ulm, Germany) containing 95% O_2 and 5% CO_2. The solutions had the following compositions.

- Krebs-Ringer (KR) contained always (mM): 118 NaCl, 0.8 $MgSO_4$, 1.0 KH_2PO_4, 11.1 Glucose, 25 $NaHCO_3$, 2.5 $CaCl_2$. pH was set at 7.4. KCl concentrations were adjusted in the range of 0 to 10 mM. At 0 mM [K^+] 1.0 KH_2PO_4 was replaced by 1.0 NaH_2PO_4.

- Bretag contained always (mM): 107.7 NaCl, 0.69 $MgSO_4$, 1.67 NaH_2PO_4, 5.05 Glucose, 26.2 $NaHCO_3$, 1.53 $CaCl_2$, 9.63 Na-gluconate, 7.6 Saccharose. pH was set at 7.4. KCl concentrations were adjusted in the range of 0 to 10 mM.
- Anthracene-9-Carboxylic Acid (9-AC): Experimental myotonia was achieved by inhibiting gCl^- with 9-AC. As a stock solution 9-AC was dissolved in dimethylsulfoxide (DMSO) which itself causes an increase in osmolarity. For this reason the optimum concentration of stock solution was prepared 500 times of the desired end concentration, i.e. 50 mM in 80% DMSO. The osmolarity of the final solutions was checked and pH was equilibrated to 7.4. 9-AC stock solution was added to the solutions to yield an end concentration of 100 μM.
- Cl^--free solution contained always (mM): 107.7 Na-methanesulphonate, 0.69 $MgSO_4$, 1.67 NaH_2PO_4, 26.2 $NaHCO_3$, 9.63 Ca-gluconate. pH was set at 7.4. The solution contained non-permeant methanesulphonate instead of chloride. K-methanesulphonate concentrations were adjusted in the range of 0 to 7 mM.
- Krebs-Ringer (KR), Bretag, and Cl^--free low and high osmolarity solutions were modified by reducing glucose content for low osmolarity solutions (250 mOsm) or adding glucose to get a specific osmolarity of various levels (300-550 mOsm).

Chemicals used in the above mentioned solutions were purchased from the following companies: Applichem (Darmstadt, Germany): NaCl; Merck (Darmstadt, Germany): KCl, $NaHCO_3$, NaH_2PO_4, $CaCl_2$, KH_2PO_4, KOH, saccharose; Sigma-Aldrich (Steinheim, Germany): 9-AC, DMSO, $MgSO_4$, glucose, HCH_3SO_3, NaOH, Na-gluconate, Ca-gluconate.

2.2.2 Pharmacological substances

In the in vitro contraction test (IVCT) and electrophysiological experiments the agents were added into the bath solution. Each substance was dissolved in a suitable solute as shown in Table 3 for a stock solution which usually contained 100-500 times higher concentration than the desired final concentration. In the dose-response experiments, the used stock solution was prepared in high concentration to avoid an increase in osmolarity of the bath solution. The suitable volume of stock solution was added directly to the organ bath step by step to obtain the desired dosage of drugs.

Aliquots were stored at -20ºC. On the day of the experiment an aliquot of the stock solution was defreezed and diluted in the bath solution. In the few cases in which a muscle bundle was exposed to multiple pharmacological substances, care was taken that

remainders of the previous agent were washed off and that the original baseline of muscle bundle tension had been regained and kept stable for at least 20 min.

Table 3: Pharmacological substances used in the study.

Substance	Stock solution	Solute	Organ bath concentration	Source
Bumetanide	100 mM	80% DMSO	50, 100 x 10^{-6} M	Sigma-Aldrich, Steinheim, Germany
Retigabine	50 mM	80% DMSO	10 - 100 x 10^{-6} M	Wyeth Ayerst Research, Saint Davids, USA
Mexiletine	50 mM	100% DMSO	10 - 70 x 10^{-6} M	Sigma-Aldrich, Steinheim, Germany
Flecainide	50 mM	100% DMSO	10 - 70 x 10^{-6} M	Sigma-Aldrich, Steinheim, Germany
NS1608	20 mM	100% DMSO	20, 25, 30 x 10^{-6} M	Neurosearch, Ballerup, Denmark
Isopimaric acid	10 mM	100% DMSO	10, 20 x 10^{-6} M	Sigma-Aldrich, Steinheim, Germany
Paxilline	10 mM	100% DMSO	10, 20 x 10^{-6} M	Sigma-Aldrich, Steinheim, Germany
XE991	10 mM	Distilled water	30 - 50 x 10^{-6} M	Tocris Bioscience, Avonmouth, UK
Caffeine	100 mM	Distilled water	1 - 4 x 10^{-3} M	Merck, Darmstadt, Germany
Halothane			1 - 4% v/v or 0.11 - 0.88 mM	Zeneca, Plankstadt, Germany

2.3 IN VITRO CONTRACTION TEST (IVCT)

2.3.1 Muscle dissection and preparation

Mice (WT and ADR) aging 45-70 days were killed by cervical dislocation after narcosis with CO_2-gas for at least 2 min in agreement with the regulations of the local animal welfare committee (Ulm University). The hindlimbs were dissected from the mouse body and, after removing the skin, they were fixed in a large Petri-dish on a thin layer of Sylgard (Dow Corning, Belgium). The dissection chamber was filled with 4.5 mM $[K^+]_o$ KR solution and was continuously bubbled with carbogen. The gastrocnemius, extensor digitorum longus (EDL), soleus muscle and diaphragm were dissected.

Human vastus lateralis muscle biopsies were obtained from two donors and one patient suffering from paramyotonia congenita. The fresh surplus tissue from caffeine-halothane contraction tests for malignant hyperthermia was also used for this study under approval of the local ethics committee (Ulm University). This tissue was surgically dissected under regional anesthesia according to the guidelines of the European Malignant Hyperthermia Group (Ording et al. 1997). The muscle bundles were dissected into 6-10 strips (length: 15-25 mm; width: 2-3 mm; weight: 120-250 mg).

2.3.2 Force measurements

Muscle strips from human (human vastus lateralis muscle) or from mice (gastrocnemius, EDL, soleus muscle and diaphragm) were mounted in a organ bath (Fig. 5). For attaching the mouse muscle to the force transducer a ring was fixed at the distal tendon and a piece of silk thread at the proximal one. The muscle was connected to the free arm of a force transducer (FT) (Model FT03, Glass Instruments, Quincy, USA). The force transducer was connected to a bridge amplifier (BA) and an analog-digital board (ADC, Digidata 1200B, Axon Instruments, Union City, CA, USA). The signal was recorded and stored in binary files in a personal computer (PC). Special software (written in Delphi 1.0, Borland international, Scotts Valley, CA, USA) had previously been developed in our laboratory for controlling electrical stimulation and the force recording (Ursu et al. 2001). A pair of platinum electrodes was placed on the lateral parts of the muscle and received the electrical pulses from the stimulator (S) that was controlled by Transistor Transistor Logic (TTL) signals from the interface board.

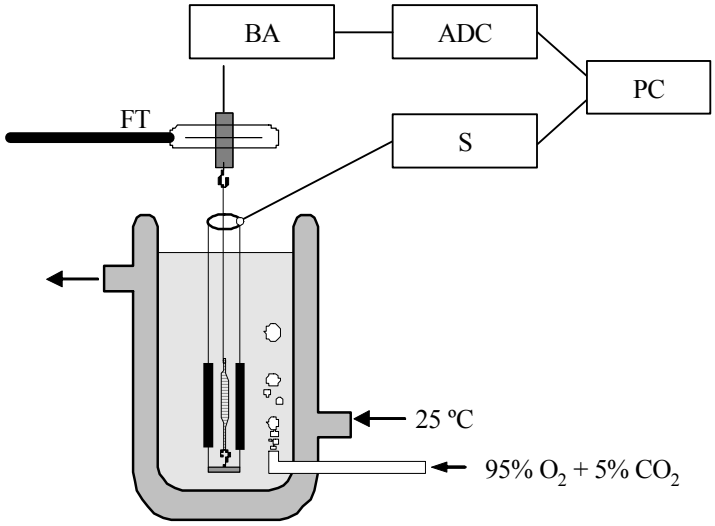


Figure 5: Scheme of the setup used for the force recordings in muscles.

FT - force transducer, BA - bridge amplifier, ADC - analog digital converter, S - stimulator, PC - computer. The solution in the organ bath was saturated with continuous bubbling and held at a constant temperature (25ºC for mouse samples, 37ºC for human muscle).

All experiments started after allowing the muscle to equilibrate in the chamber solution for a period of at least 15 min. A supramaximal stimulus (25 V) with a pulse duration of 1 ms was used in all protocols. For determining the optimum prestretch, test pulses were applied and the twitch force production was monitored. The optimal length was set to the value where the twitch force showed the maximum amplitude, but it was not stretched over 150% of its initial length that was measured before testing. The data sampling frequency was 250 Hz for single twitch stimulation and 1 kHz for tetanic stimulation. Data sampling was synchronized with the output of TTL pulses that triggered the analog device used for stimulation. Similar conditions were used for human vastus lateralis muscle, which was dissected as described above. The muscle strip was secured with silk sutures to a fixed point at both ends of a strip and then mounted in the recording chamber.

2.3.2.1 Single twitch stimulation

Single twitches were stimulated at a frequency of 0.1 Hz. For each muscle a set of 20 successive twitches were recorded.

The parameters that were evaluated were: force (mN) - twitch tension, as the maximum force amplitude, T_{peak} (ms) - time to peak, measured from the beginning of the pulse until

the twitch reached maximum amplitude, $T_{1/2}$ (ms) - time back to half peak, determined as the time between the peak and the time when the force has decreased to it's half value. $T_{90/10}$ (ms) - time from 90% to 10% of peak determined as the time between 90% of peak and the time when the force has decreased to its 10% value (Fig. 6).

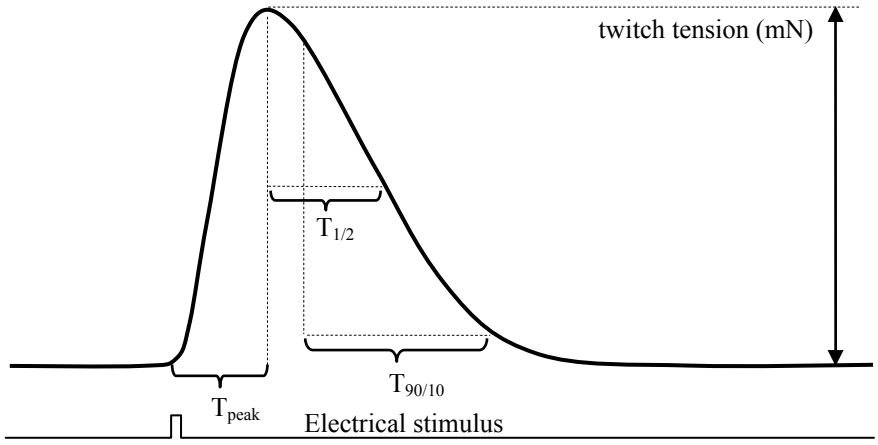


Figure 6: Scheme of a typical twitch recording with the corresponding parameters used for analysis.

The electrical stimulus has a duration of 1 ms and a voltage of 25 V. T_{peak} is a marker for the steepness of the upstroke of a single twitch. The relaxation times were measured as $T_{1/2}$ and $T_{90/10}$. For myotonic muscle $T_{90/10}$ usually gives more robust results than $T_{1/2}$.

2.3.2.2 Tetanic stimulation

To characterize the contractile behavior a common protocol of the force-frequency relation was used for inducing tetanic contraction (Westerblad et al. 1993). Different stimulation frequencies were applied for a fixed duration of 500 ms and the maximal force during each train was recorded. Frequencies were varied in 10 Hz steps from 50 to 120 Hz for EDL and diaphragm muscle and from 40 to 100 Hz for soleus and gastrocnemius muscle. A set of two consecutive tetani were recorded for each muscle, with an interval of 60 s between two successive recordings.

To characterize the transient weakness and respectively the warm-up phenomenon in myotonia, the tetanic stimulation was prolonged to 4 s. The full warm-up frequency in myotonic muscle was determined as the frequency at which the relaxation deficit and transient weakness were absent.

The measured parameters were: tetanic tension (mN) - maximum force amplitude, $T_{1/2,\ contract}$ (ms) - time to half of maximal force, $T_{1/2,\ relax}$ (ms) - time from end of the last pulse to half relaxation (Fig. 7).

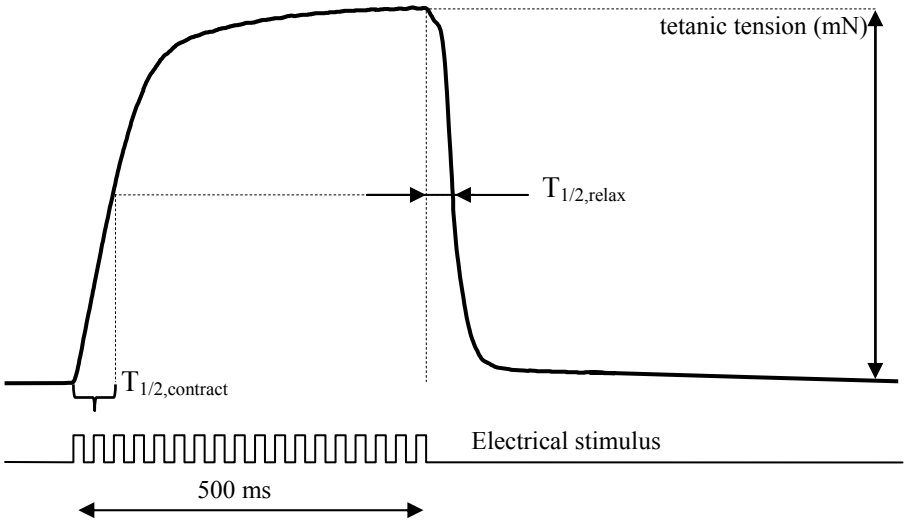


Figure 7: Scheme of a typical tetanus with the corresponding parameters used for analysis.

Tetanic contraction was stimulated using a train of electrical stimuli with a defined frequency for a duration of 500 ms. $T_{1/2}$, contract was measured from the start of the stimulus to the time when contraction reached half of the maximum tetanic tension; $T_{1/2}$, relax was measured from the end of stimulation to half of the maximum tetanic tension.

2.3.3 Caffeine and halothane contracture

To investigate the effects of Ca^{2+} release from SR through ryanodine receptors type 1 (RyR1), the RyR1 activators caffeine and halothane were tested (Ording et al. 1997). The apparatus used for experiments involved the use of four glass recording chambers (Sauer-Feinmechanik, Wuerzburg, Germany) with force-voltage transducers (RS Biomed Tech, Sinzig, Germany). Muscle bundles were dissected from control and ADR mice as described in the previous section. The strips were secured with silk sutures and connected with a force displacement transducer. The specimen were immersed in 20 ml of a 4.5 mM $[K^+]_o$ KR solution in the recording chambers and electrically stimulated (frequency 0.2 Hz, pulse duration 1 ms, supramaximal voltage). The bath solution was saturated with continuous bubbling and held at a constant temperature of 25°C for murine samples and at 37°C for human samples. Mechanical tension was recorded while increasing

concentrations of the test agents were applied. Stock solution (100 mM) of caffeine was added to the tissue bath to yield concentration steps of 1, 2, 3 and 4 mM. Halothane was applied, using a vaporizer (Vapor 19.1 Draeger, Luebeck, Germany), to the tissue bath in increasing concentration from 1 to 2, 3, and 4% v/v. The concentration steps were held for 3-6 min. Contracture curves were displayed and recorded with a computer based data–evaluation program.

2.4 ELECTROPHYSIOLOGICAL METHODS

2.4.1 Muscle tissue preparation

45-70 days old mice (WT and ADR) were killed by direct exposure to CO_2-gas, followed by cervical dislocation, in agreement with the regulations of the local animal welfare committee. The diaphragm was quickly removed and placed in a dissection chamber filled with 4.5 mM $[K^+]_o$ KR solution. The hemidiaphragm was then dissected out from the tendon to ribs without injuring the muscle fibers and was then cut along the muscle fibers to 7 mm width.

2.4.2 Internal microelectrode

The recording chamber, the microscope and the manipulators were placed on an anti-vibration table. All metal parts near the head-stage were grounded.

Glass microelectrodes were pulled on a horizontal two stage puller (DMZ Universal Puller, Zeitz Instruments, Munich, Germany) from thin wall borosilicate glass pipettes with filament (1.5 mm OD, 1.05 mm ID; Science Products, Hofheim, Germany). Sharp glass electrodes were pulled in a way to minimize the access resistance and cell damage. Electrodes were filled with 3 M KCl and had a resistance of 10 ± 3 MΩ. A silver wire (length = 5 cm, diameter = 300 µm, Science Products, Hofheim, Germany) was chemically chlorated in Fe_2Cl_3 solution to obtain Ag/AgCl electrodes. The recording chamber was placed under a microscope (Stereomicroscope, Nikon SMZ-U, Dornach, Germany). Head-stages and pipette holders were fixed on Leitz manipulators (Wetzlar, Germany). The feedback amplifier Axopatch 200B was used for all membrane potential measurements. The voltage amplifier output was digitized by a Digidata 1200 AD/DA converter, connected to a personal computer running fetchex 6.0 pClamp acquisition software. The equipment was obtained from Axon Instruments, Union City, USA. The membrane potential was recorded on the hard drive and additionally a hard copy record was made for each protocol. Analysis was

done on a PC using special evaluation software (pClamp 8.0, Axon Instruments, Union City, USA).

2.4.3 Membrane potential measurements

All the experiments were performed at room temperature (20ºC). A 7 mm wide hemidiaphragm prepared as described above was mounted in a chamber containing isoosmolar 4.5 $[K^+]_o$ KR solution. The bath solution was buffered to pH = 7.4 by bubbling with carbogen (95% O_2, 5% CO_2). The small volume of the chamber of about 500 µl allowed a rapid exchange of the solution. The chamber solution was changed according to a protocol similar to the IVCT experiments. Some solutions contained 500 nM of the $Na_v1.4$ channel blocker tetrodotoxin (TTX; Latoxan, Valence, France), in order to block the electrical activity of repetitive AP bursts in myotonic muscle. The muscle fibers were impaled with sharp glass microelectrodes after allowing the muscle to equilibrate in the chamber solution for a period of at least 15 min. A pipette was mounted in the holder that contained a Ag/AgCl electrode and a connector to plug into the headstage of the amplifier. For the bath electrode an Ag/AgCl pellet was used. After applying a test solution, the pipette offset potential was first compensated. The amplifier was switched to current clamp mode at zero net current. Then, the membrane potential was continuously recorded by an acquisition system in parallel with stepwise penetration of the fibers by the glass electrode. The bundle was impaled several times at different transversal positions (Fig. 8).

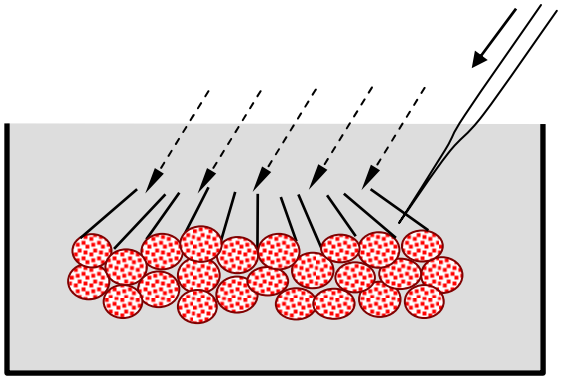


Figure 8: Schematic representation of the impalement procedure for membrane potential recording in fibers bundle.

The microelectrode was filled with KCl 3M and was impaled against the fiber bundle at different positions (arrows).

2.5 STATISTICS

Unless otherwise stated, averaged data are presented and plotted as means ± standard error of the mean (SEM) (n = number of experiments). Wilcoxon non-parametric tests were used to test for significant differences of mean values. A significance level of $p < 0.05$ was applied.

3 RESULTS

3.1 MECHANOGRAPHIC REGISTRATIONS

3.1.1 Contraction behavior of ADR muscle

3.1.1.1 Single twitch analysis

To characterize the myotonic activity, force measurements were carried out on muscle bundles from ADR mice and pharmacologically induced myotonia in WT mice. Fig. 9 shows a sample recording in ADR muscle. The myotonic contraction had a slowed relaxation, which is represented by the $T_{90/10}$ parameter. This behavior is the in vitro correlate of the relaxation deficit in low gCl^- myotonia. Superimposition of single twitches caused a force build-up. After a few twitches, the relaxation time normalized and the classical warm-up phenomenon occurred.

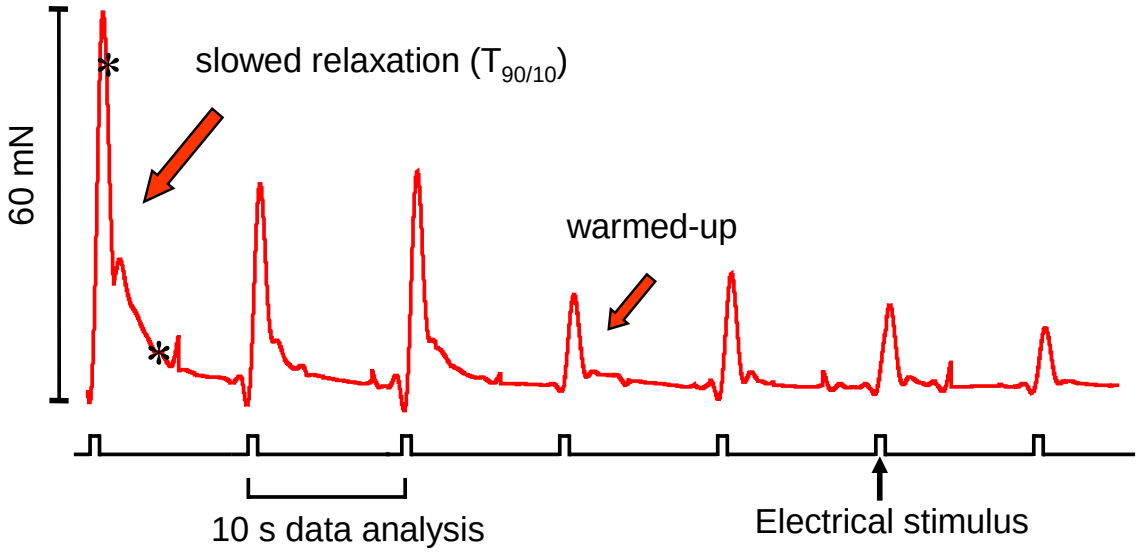


Figure 9: A representative in vitro force registration of ADR gastrocnemius muscle.

A bundle was electrically stimulated with 1 ms pulses at 0.1 Hz in 5 mM $[K^+]_o$ Bretag at 25 ºC. The myotonic reaction appeared as a slowed relaxation ($T_{90/10}$) in some first twitches. At twitch 4 the relaxation time normalized and a classical warm-up phenomenon was observed. The high twitch force seen in some first twitches was caused by a summation effect. * indicates 90% and 10% of the maximum force.

3.1.1.2 Tetanic stimulation

The tetanus frequency was determined in WT and myotonic muscle. The maximal tension at the end of a fixed stimulation period (500 ms) was recorded. The frequency of stimulation was varied in 10 Hz steps from 60 to 120 Hz in EDL and diaphragm and from 40 to 100 Hz for soleus and gastrocnemius muscle. Two consecutive tetani were recorded at an interval of 60 s for each frequency (Fig. 10).

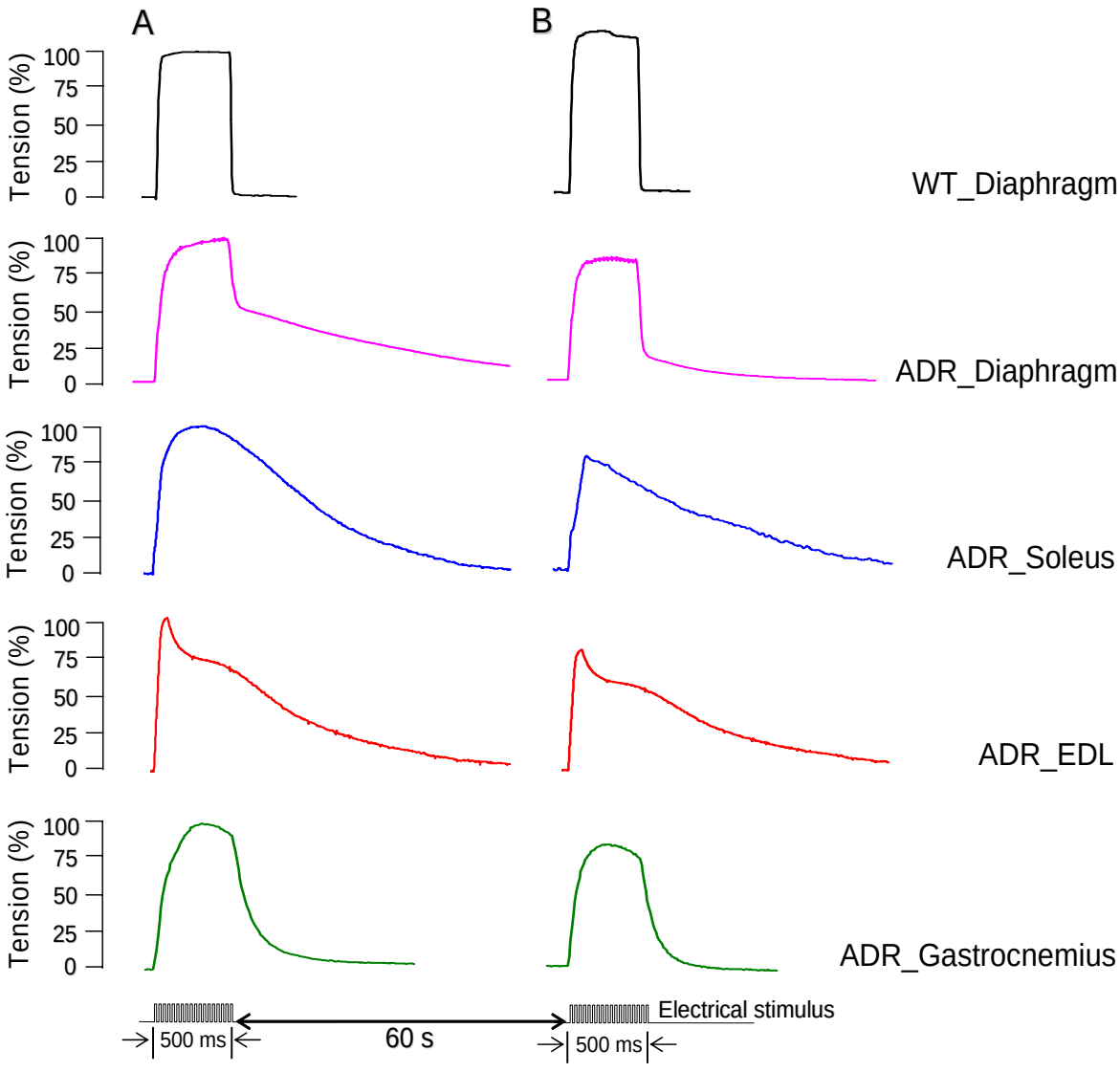


Figure 10: Two consecutive tetanic contractions recorded at full tetanus frequency.

Example records of the 1st (A) and 2nd tetanus (B) in ADR muscle. All ADR samples showed a slowed relaxation and a decrease of tetanic force (weakness) in the 2nd train.

As expected, myotonic muscle demonstrated a slowed relaxation from tetanic contraction. The full tetanus frequency was determined as the frequency at which maximum force was reached. The results obtained from several experiments in WT, ADR, and 9-AC muscle are summarized in Table 4. The full tetanus frequency in myotonic muscle was significantly lower compared to WT. Another difference of myotonic samples in comparison to control muscle is a drop of force at the second tetanic stimulation (Table 4/ Fig. 10). In patients suffering from low gCl^- myotonia this phenomenon is known as transient weakness.

Table 4: Full tetanus frequency in control and myotonic muscle.

Model	Muscle	Tetanus frequency (Hz)	Force of 2nd tetanus relative to 1st tetanus (% of initial tension)
WT	Soleus (n=3)	76.8 ± 2.7	>100
	Gastrocnemius (n=4)	80.0 ± 0.0	>100
	EDL (n=4)	107.5 ± 4.2	>100
	Diaphragm (n=2)	105.0 ± 3.5	>100
ADR	Soleus (n=3)	50.0 ± 4.7	82.0 ± 0.2
	Gastrocnemius (n=5)	60.0 ± 5.7	84.7 ± 2.7
	EDL (n=5)	75.0 ± 2.0	82.6 ± 5.6
	Diaphragm (n=4)	50.0 ± 3.5	88.3 ± 1.3
9-AC	Soleus (n=3)	50.0 ± 2.0	99.5 ± 0.1
	EDL (n=2)	75.0 ± 3.5	93.9 ± 0.8

To study further the transient weakness, tetanic stimulation was prolonged to 4 s. As shown in Fig. 11 the force of ADR diaphragm was not sustained after approximately 500 ms stimulation but fell quickly to a plateau. By continuous stimulation the force gradually increased again. The transient weakness was more prominent at lower frequencies and it was muscle type dependent. Soleus muscle showed the most prominent loss of force, which is a hint for the potential involvement of type IIa (oxidative) fibers (Table 5).

At higher frequencies the transient weakness and relaxation deficit were absent reflecting the warm-up state. However, in the warm-up state the force level did not always reach the values of the initial tetanic contraction. These results confirm the clinical observation that myotonic stiffness in low gCl^- conditions can be eased by repetitive voluntary contractions.

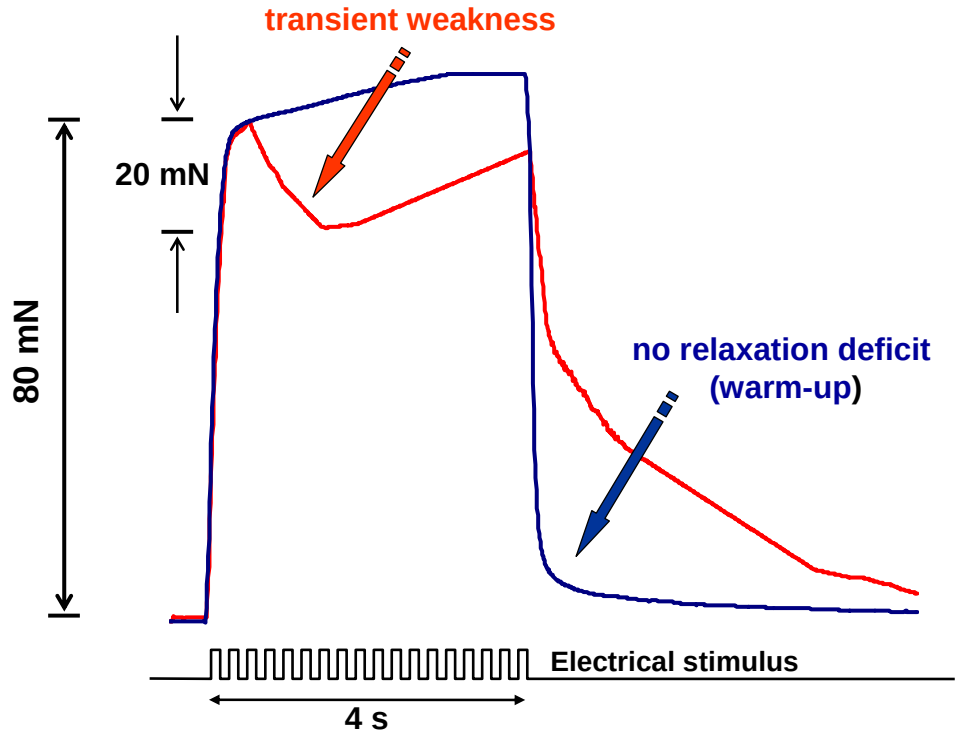


Figure 11: Transient weakness in ADR- myotonic muscle

Electrical stimulation of ADR-diaphragm at 50 Hz presented transient weakness (red curve). The transient weakness lasted approximately 3-4 s. Stimulation with the frequency of full warm-up at 100 Hz showed no relaxation deficit and no transient weakness (blue curve).

Table 5: Transient weakness and frequency of full warm-up in ADR-myotonic muscle.

Muscle	Tetanus frequency (Hz)	Transient weakness		Frequency of full warm-up
		mN	%	
Soleus (n=3)	50.0 ± 4.7	7.4 ± 0.1	44.0 ± 0.2	73.3 ± 2.7
Gastrocnemius (n=5)	60.0 ± 5.7	32.4 ± 9.3	24.0 ± 4.6	87.5 ± 2.2
EDL (n=5)	75.0 ± 2.0	5.4 ± 0.7	31.9 ± 3.9	97.5 ± 4.2
Diaphragm (n=4)	50.0 ± 3.5	23.1 ± 2.0	22.1 ± 2.0	97.5 ± 2.2

3.1.2 $[K^+]_o$ effects

3.1.2.1 Influence of $[K^+]_o$ on myotonic activity

To investigate the influence of $[K^+]_o$ on the myotonic activity, $[K^+]_o$ in the bath solution was varied between 1 to 10 mM. Contractions and relaxation parameters of 20 consecutive twitch contractions were recorded. Fig. 12 displays representative traces of force registrations of mouse gastrocnemius muscle from the WT and ADR. At physiological conditions (3.5 mM $[K^+]_o$), the relaxation time of twitch response in WT muscle kept stable over 20 consecutive twitches (Fig. 12A). In myotonic muscle the relaxation time was prolonged (Fig. 12B).

The systematic evaluation from several experiments performed under the mentioned conditions is displayed in Fig. 13 and 14. The average relaxation time ($T_{90/10}$) at the various $[K^+]_o$ is plotted in Fig. 13. Myotonic muscle presented with high $T_{90/10}$. An increase of $[K^+]_o$ led to a reduction of the relaxation time ($T_{90/10}$ and $T_{1/2}$). This effect was concentration dependent. In 9-AC and Cl^--free myotonia the relaxation time reached the control level at 7 mM $[K^+]_o$. In ADR mice the myotonic activity disappeared at 10 mM $[K^+]_o$. In summary, high $[K^+]_o$ acts antimyotonic by normalizing the relaxation time.

Another finding is a reduction of twitch force at high $[K^+]_o$. Twitch force was analyzed in the warm-up state in order to avoid a summation bias of superimposed myotonic twitches (Fig. 14). At physiological conditions, myotonic muscle had a lower force than WT muscle, particularly in ADR muscle. The average value (20.9 $\pm$ 2.1 mN) was lowered by a factor of 2.6 in comparison to WT (55.1 $\pm$ 5.6 mN).

Lowering $[K^+]_o$ to 1 mM did not significantly change the twitch force in the murine tissue. However, withdrawing K^+ from KR solution decreased the twitch force in human tissue obtained from the healthy donors. Exposing the vastus lateralis strip to K^+-free KR caused a significant decrease of twitch force when compared to 4.5 mM K^+ KR. The average values were 30.8 $\pm$ 3.5 mN (K^+-free KR, n=9) and 44.5 $\pm$ 3.3 mN ($[K^+]_o$ = 4.5 mM, n=20). Moreover, $[K^+]_o$ values above the physiological range led to a decrease of twitch force in WT and all models of myotonia.

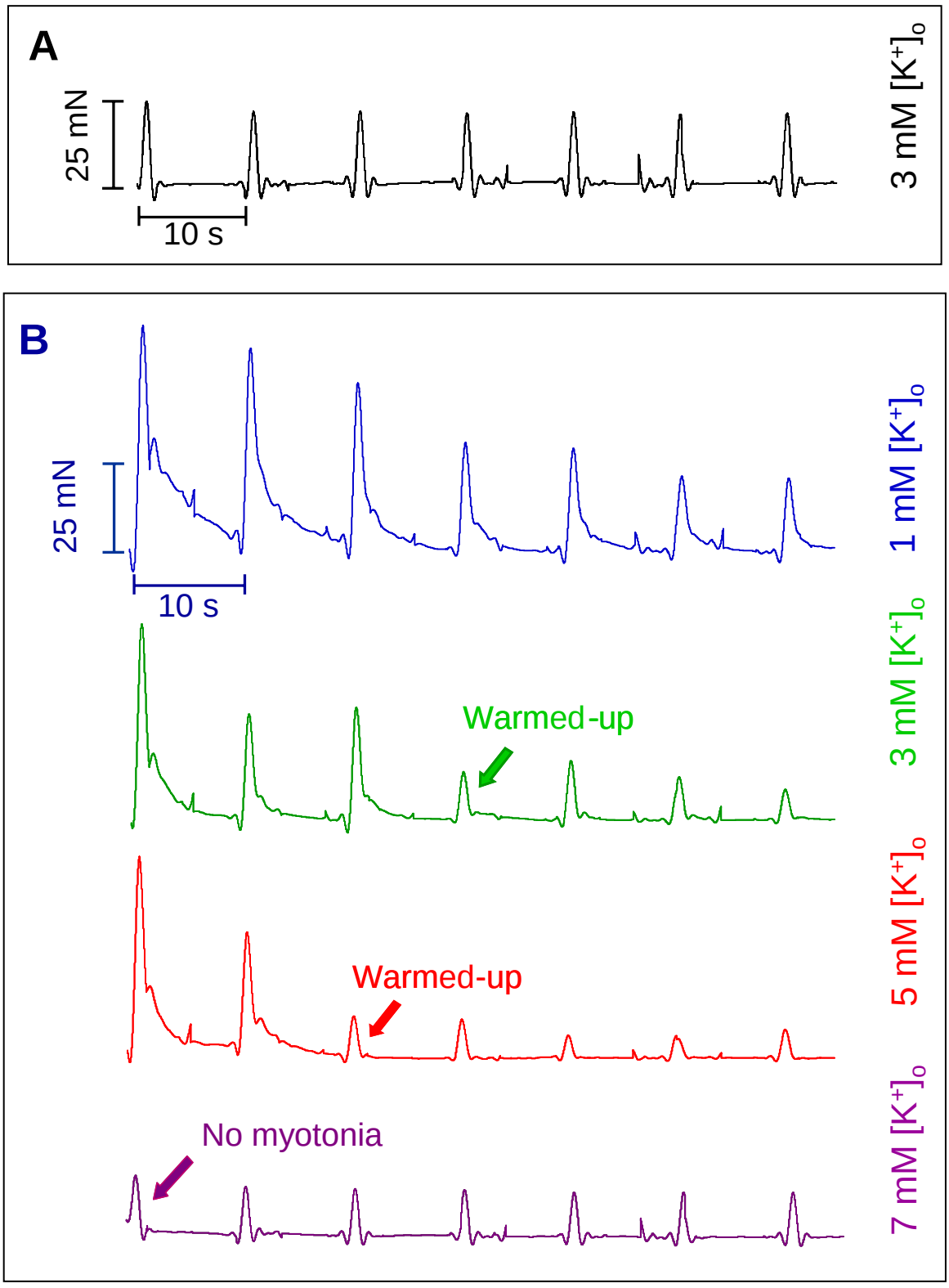


Figure 12: Influence of $[K^+]_o$ on the myotonic activity.

Representative mechanographic registrations of gastrocnemius muscle in a control (A) and a ADR strip (B) are displayed. The strip was electrically stimulated with 1 ms pulses at 0.1 Hz after 20 min incubation in 1, 3, 5 and 7 mM $[K^+]_o$ Bretag solution. Force was increased due to a superimposition in some first twitches of contraction in ADR muscle. Myotonic activity was reduced with increasing $[K^+]_o$. As well, the warm-up phenomenon was $[K^+]_o$ dependent.

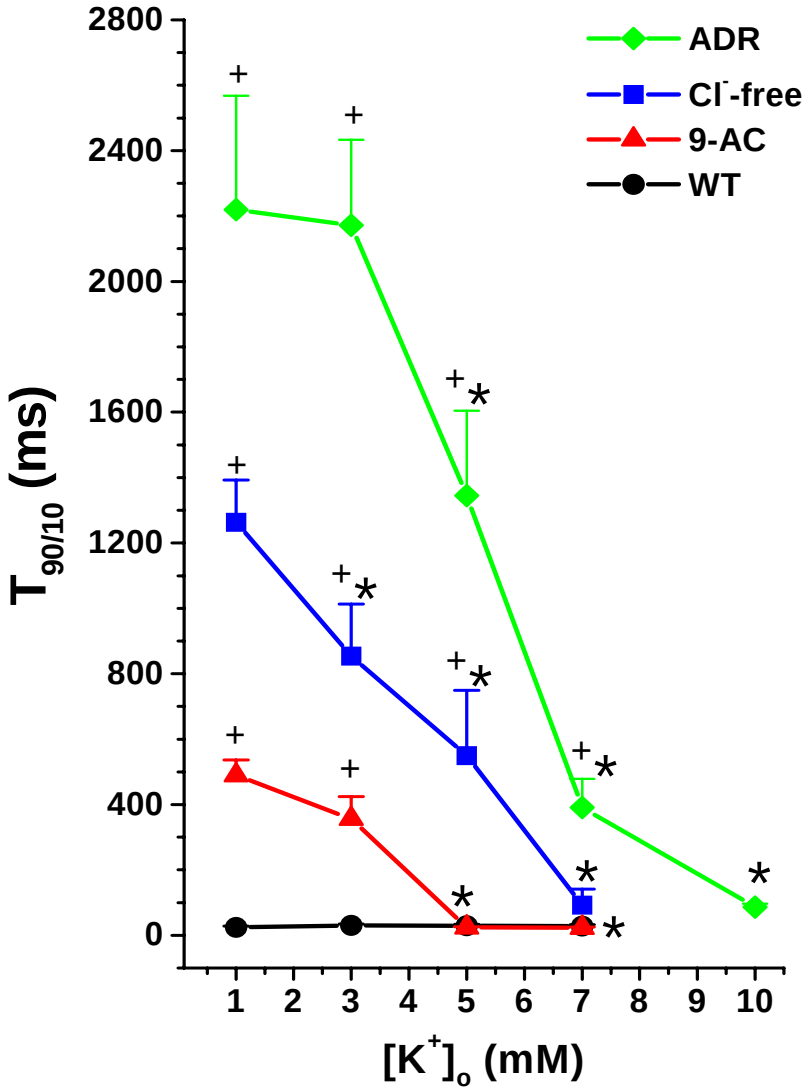


Figure 13: Influence of $[K^+]_o$ on the relaxation time ($T_{90/10}$).

High $[K^+]_o$ dose-dependently reduced $T_{90/10}$ in 9-AC (n=12), Cl^--free (n=12) and ADR-myotonic muscles (n=14). WT data were obtained from 15 muscle strips. Symbols represent mean values of 20 single twitches. + significant difference vs. control. * significant difference vs. 1 mM $[K^+]_o$.

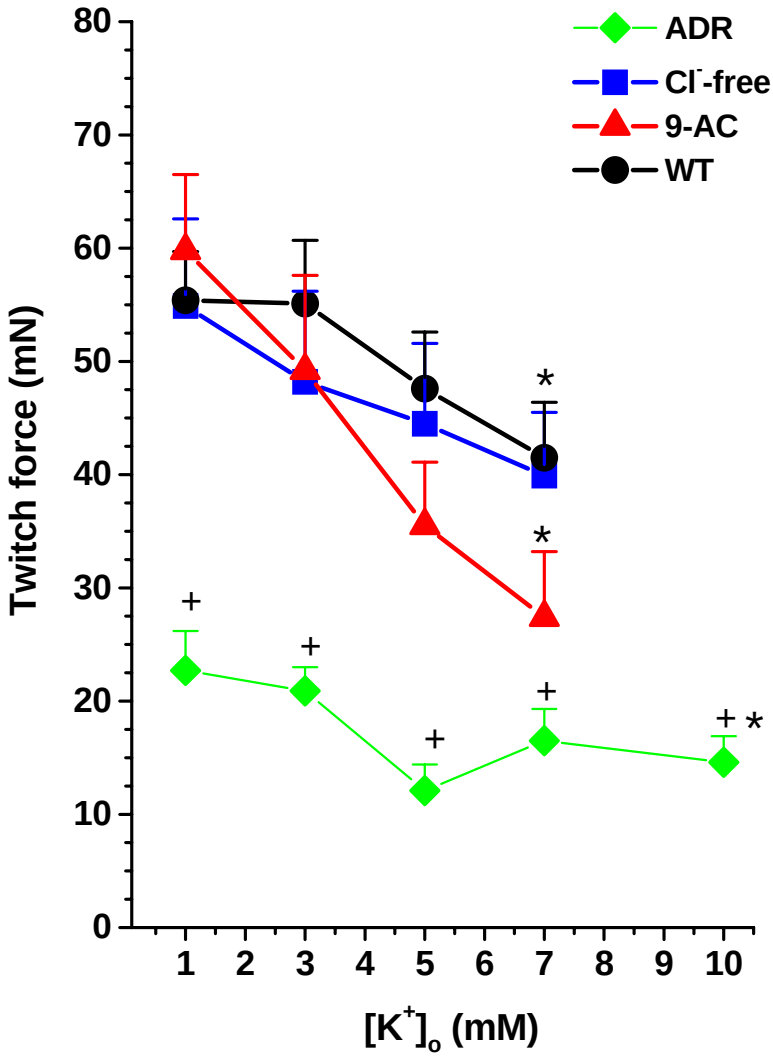


Figure 14: Influence of $[K^+]_o$ on the twitch force.

Increasing $[K^+]_o$ went along with a reduction of twitch force in 9-AC (n=12), Cl^--free (n=12) and ADR-myotonic muscles (n=14). The shown data were evaluated from muscle strips in the warm-up state. A significant decrease of twitch force was also observed in WT muscle (n=15) at 7 mM $[K^+]_o$. Symbols represent mean values of the last 10 twitches of contraction. + significant difference vs. control. * significant difference vs. 3 mM $[K^+]_o$

3.1.2.2 Facilitation of the warm-up phenomenon in high $[K^+]_o$

Fig. 15 represents the results from WT and various models for low gCl^- myotonia. Besides $T_{90/10}$, also half relaxation time ($T_{1/2}$) showed the myotonic relaxation deficit. This emphasizes the experimental robustness of the various myotonia models in the potassium challenge.

In control, $T_{1/2}$ kept nearly stable over 20 consecutive contractions. $T_{1/2}$ was increased by a factor of roughly 100 in some first twitches of ADR muscle. This reflects the strong myotonic activity in ADR mice. After some twitches, the warm-up phenomenon emerged and $T_{1/2}$ reached control levels. The warm-up phenomenon of ADR, 9-AC and Cl^- -free myotonic muscle appeared at twitch 13.0 ± 1.9, 7.3 ± 0.8, 7.5 ± 1.9, respectively.

With increasing $[K^+]_o$ to 5 mM the warm-up effect started at earlier twitches (6.0 ± 0.8 for ADR, 1.1 ± 0.1 for 9-AC and 2.9 ± 0.5 for Cl^--free). In 7 mM $[K^+]_o$ myotonia was completely suppressed in 9-AC and Cl^--free myotonic models. ADR preparations still showed a weak myotonia at this concentration.

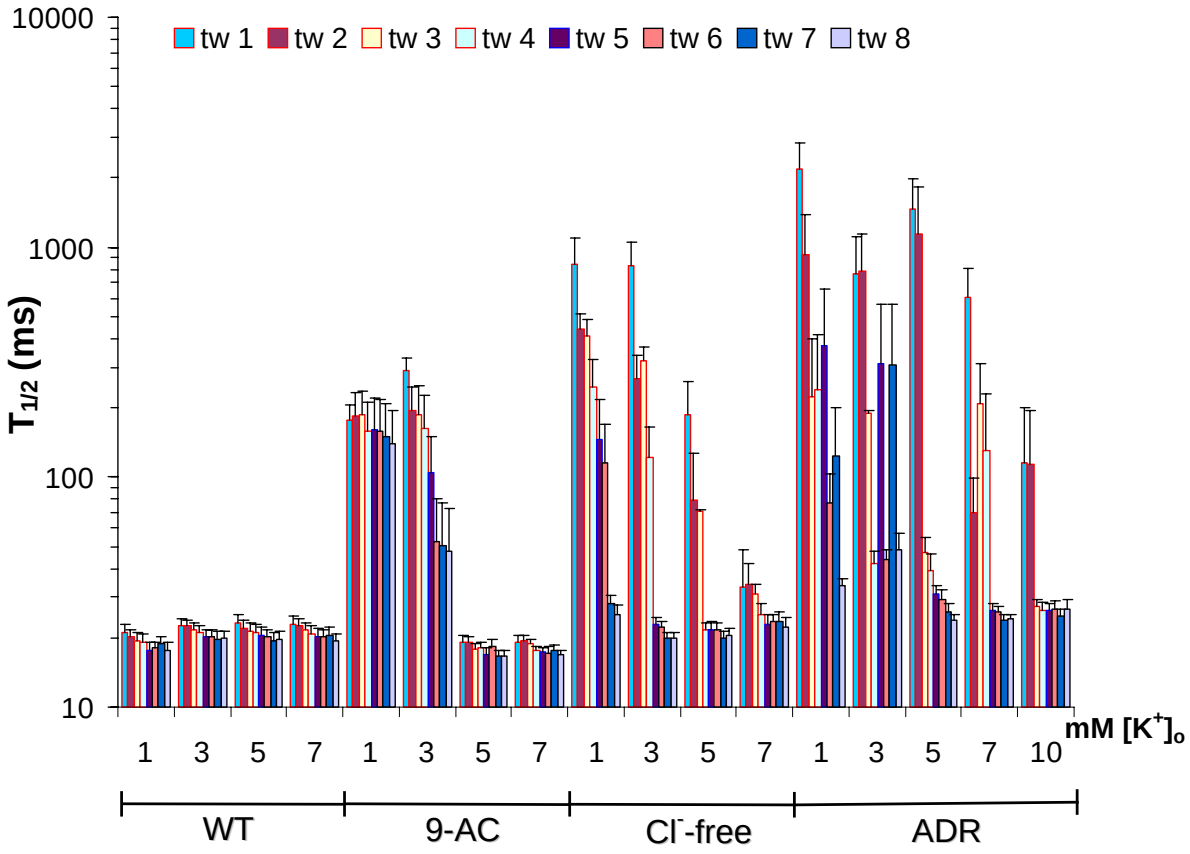


Figure 15: Influence of $[K^+]_o$ on the warm-up phenomenon.

Data are the average $T_{1/2}$ of the first 8 twitches of contraction obtained from WT (n=15), 9-AC (n=12), Cl^--free (n=12) and ADR-muscles (n=14). K^+ concentration-dependently facilitated the warm-up phenomenon in all models of myotonia.

3.1.3 Time dependent $[K^+]_o$ effects on myotonia

The K^+ gradient plays an important role in T-tubular repolarization. However, the tight opening and long narrow shape hinder the diffusion between inner and the outer extracellular space. To assess potential effects of K^+ diffusion respectively effusion, ADR-gastrocnemius muscle was incubated in 2 and 7 mM $[K^+]_o$ Bretag for 20, 120 and 240 min duration.

The contraction (T_{peak}) and relaxation times ($T_{90/10}$) were significantly lower in 7 mM $[K^+]_o$ preparations when compared to 2 mM $[K^+]_o$ (Fig. 16). These results confirm the above described antimyotonic effects of high $[K^+]_o$ solutions.

The upstroke of the twitch was faster (lower T_{peak}) at 20 min in 2 mM $[K^+]_o$ when compared to later time points. This result could be an effect of K^+ effusion out of T-tubules in due time during repetitive stimulation. On the other hand, in high $[K^+]_o$, lower T_{peak} was observed at 120 min when compared to at 20 min. The reason might be diffusion of K^+ into T-tubules during long incubation. However, the relaxation times were nearly unaffected by long incubation.

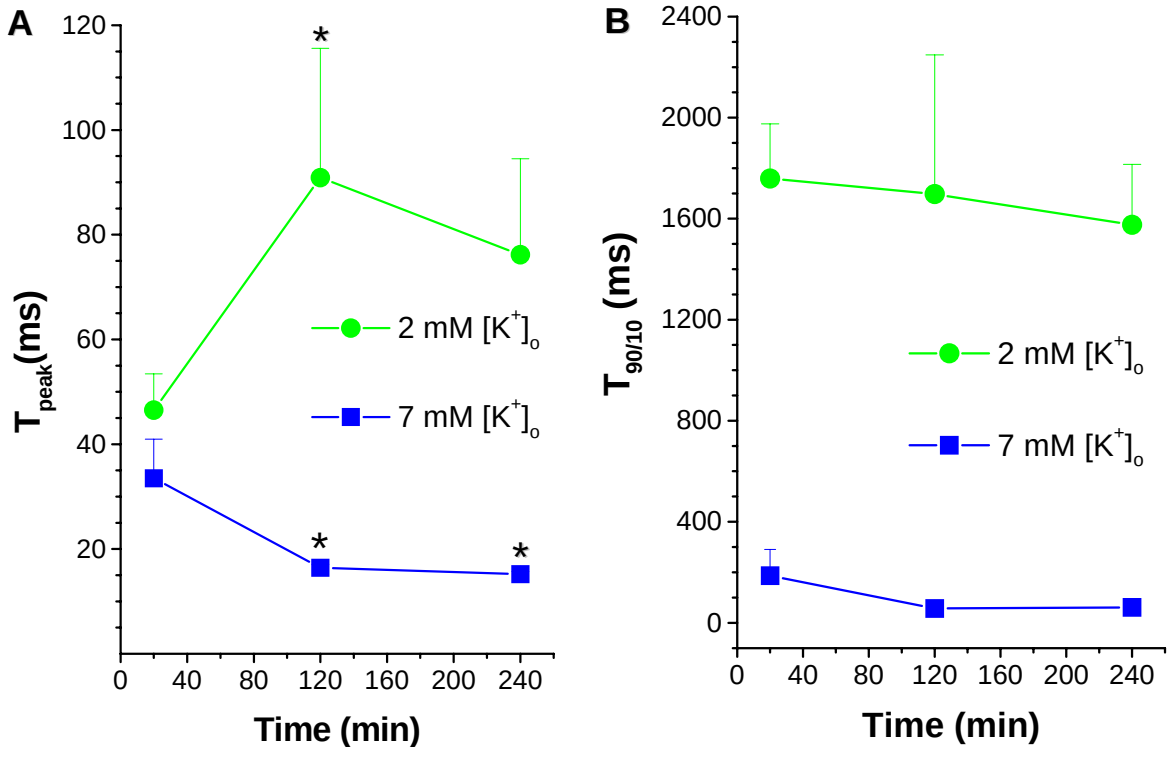


Figure 16: Time dependence of K^+ gradient on T_{peak} and $T_{90/10}$.

20 twitches were recorded in ADR gastrocnemius muscle after 20, 120, and 240 min incubation in 2 mM and 7 mM $[K^+]_o$ Bretag solution (n=8). Symbols represent mean values of 20 single twitches of T_{peak} (A) and $T_{90/10}$ (B). * significant difference vs. 20 min.

3.1.4 Antimyotonic effects of elevated osmolarity

3.1.4.1 Influence of osmolarity on the myotonic activity

Another candidate mechanism for the warm-up phenomenon is a use dependent change of osmolarity. To explore the hypothesis, experiments were performed under hyperosmotic conditions (Fig. 17).

In contrast to WT muscle, the contractions of 9-AC, Cl^--free and ADR muscle showed relaxation deficits ($T_{90/10}$) at physiological conditions (300 mOsm). Under hypoosmotic conditions (250 mOsm) $T_{90/10}$ was more than 100 times longer in ADR muscle than in WT. Hypoosmotic conditions which occur during fasting are worsening the myotonic relaxation deficit.

Elevating extracellular osmolarity did not alter the relaxation time in WT. Surprisingly, raising osmolarity shortened the $T_{90/10}$ in all models of myotonia. At 400 mOsm, the relaxation time of myotonic muscle nearly reached the control level.

The twitch force in WT and myotonic muscle was diminished with increasing extracellular osmolarity. WT, 9-AC and Cl^--free muscle revealed a significant reduction of force starting at 350 mOsm. The mean values compared to the physiological osmolarity were 56.0 $\pm$ 5.6 mN vs. 74.7 $\pm$ 6.1 mN, 67.0 $\pm$ 3.9 mN vs. 79.4 $\pm$ 5.2 mN and 49.4 $\pm$ 5.0 mN vs. 66.1 $\pm$ 6.2 mN, respectively. In ADR muscle, the reduction of twitch force was significant at 550 mOsm. The twitch force was 6.5 $\pm$ 0.7 mN in comparison to 21.1 $\pm$ 3.0 mN at 300 mOsm.

This result is confirmed by experiments on human muscle obtained from human healthy donors. Exposing isolated vastus lateralis muscle to 340 mOsm decreased the twitch force. The average value was 25.8 $\pm$ 3.9 mN (n=6) in comparison to the control which was 44.5 $\pm$ 3.3 mN (n=20).

In summary, myotonic activity was alleviated under hyperosmotic conditions.

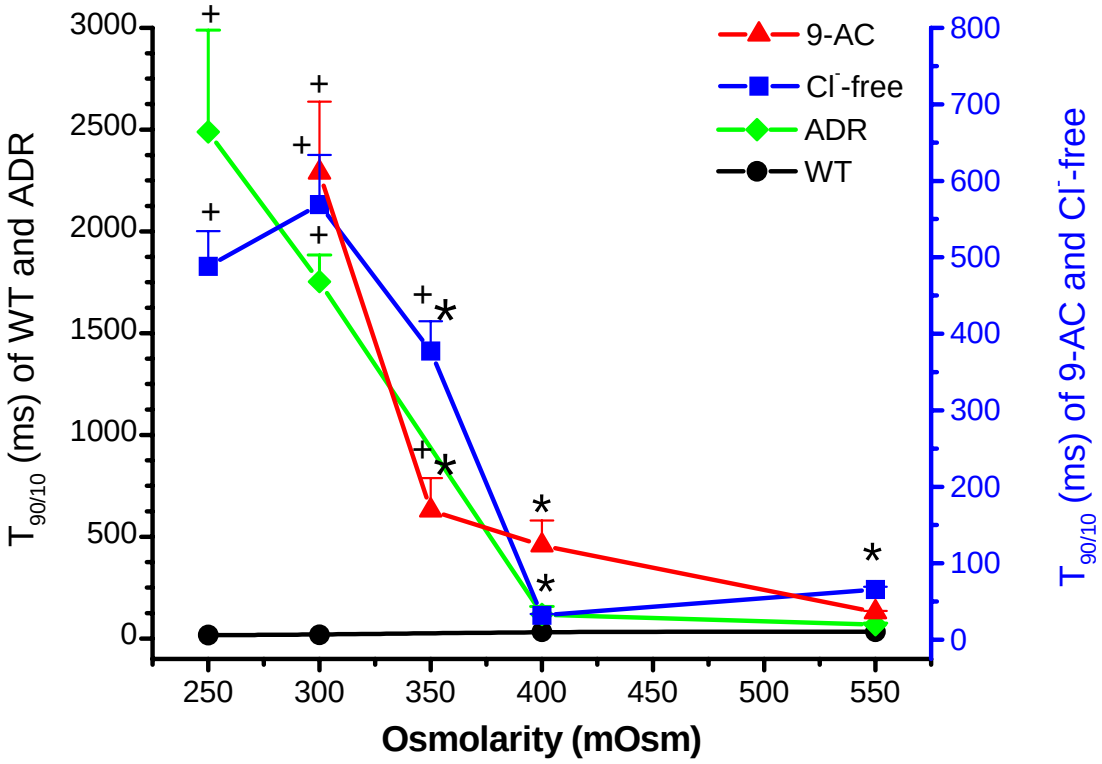


Figure 17: Antimyotonic effects of high osmolarity.

The left ordinate in black covers the average relaxation time ($T_{90/10}$) of 20 twitches in WT (n=2), and ADR (n=13) muscle. The right ordinate in blue covers the average $T_{90/10}$ of 9-AC (n=15) and Cl^--free (n=17) muscle. Increasing extracellular osmolarity significantly decreased $T_{90/10}$ in all models of myotonia. + significant difference vs. control. * significant difference vs. 300 mOsm.

3.1.4.2 Facilitation of the warm-up phenomenon by high osmolarity

High osmolarity did not only reduce $T_{90/10}$ but also had an impact on the warm-up phenomenon (Fig. 18). From 20 consecutive twitches, $T_{90/10}$ of WT muscle kept stable over the series of contractions. Contrarily, the contractions of 9-AC, Cl^--free and ADR muscle showed relaxation deficits in several first twitches at physiological osmolarity. The full warm-up twitch occurred after 20 twitches in 9-AC and Cl^--free solution and at twitch 13.0 $\pm$ 3.7 in ADR. The strong myotonic activity in ADR clearly was illustrated by an increase of $T_{90/10}$ by a factor of 500 in some twitches.

Elevating osmolarity led to an earlier onset of the warm-up phenomenon in all models of myotonia. At 400 mOsm, the full warm-up phenomenon was reached at twitch 1.17 $\pm$ 0.11, 5.14 $\pm$ 1.01 and 3.0 $\pm$ 0.53 in Cl^--free, 9-AC, and ADR muscle. These data indicate that an elevated osmolarity facilitates the warm-up phenomenon in low gCl^- myotonia.

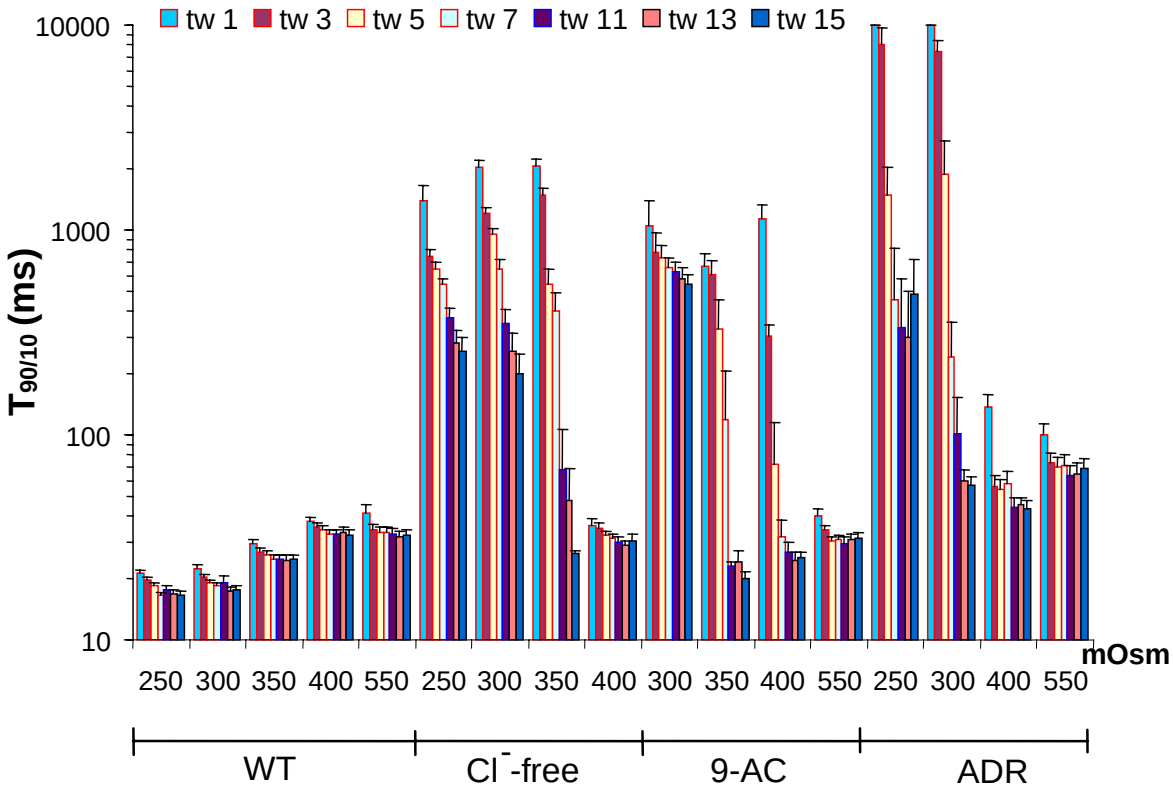


Figure 18: Influence of osmolarity on the warm-up phenomenon.

Data were obtained from the first 15 twitches of contraction of WT (n=31), Cl^--free (n=30), 9-AC (n=15), and ADR-muscles (n=13). High osmolarity facilitates the warm-up phenomenon.

3.1.4.3 Role of NKCC1 in low gCl^- myotonia

As described above, high osmolarity acted antimyotonic and promoted the warm-up phenomenon. Under hyperosmotic conditions, the cation cotransporter NKCC1 is activated and plays a major role in volume regulation (Lindinger et al. 2002). This insight led to an examination of NKCC1 in myotonic muscle. Experiments were conducted using the potent NKCC1 inhibitor bumetanide (Fig. 19). At physiological osmolarity, application of bumetanide to WT muscle did not alter the relaxation time ($T_{90/10}$). Similarly, this effect was observed in human vastus lateralis specimen.

However, under hyperosmotic conditions, bumetanide significantly prolonged the contraction in control muscle ($T_{90/10}$ = 53.7 $\pm$ 2.6 ms at 550 mOsm versus $T_{90/10}$ =33.5 $\pm$ 2.0 ms at 300 mOsm). In all models of myotonia, the antimyotonic effect of hypertonicity was at least partially antagonized by application of bumetanide. In ADR muscle bumetanide increased $T_{90/10}$ to values at physiological osmolarity.

The effect of bumetanide on the twitch force was analyzed. Bumetanide caused a non-significant increase of twitch force in control muscle obtained from murine and human samples at physiological conditions. However, under hyperosmotic conditions (550 mOsm), a significant increase of twitch force was observed. The twitch force compared to before administration was 87.7 ± 3.7 mN vs. 25.4 ± 3.2 mN.

Moreover, application of bumetanide to various models of myotonic muscle under hyperosmotic conditions significantly increased the twitch force. Twitch force most likely due to a summation effect (see 3.1.1.1). The average twitch forces at 400 mOsm with and without bumetanide to 9-AC, Cl^--free and ADR muscle were 70.7 ± 13.6 mN vs. 39.0 ± 4.3 mN (n=8), 37.1 ± 5.4 mN vs. 29.7 ± 4.3 mN (n=7), 35.5 ± 5.8 mN vs. 15.4 ± 1.8 mN (n=9), respectively.

It can be summarized that NKCC1 plays a major role mediating the antimyotonic effects of high osmolarity.

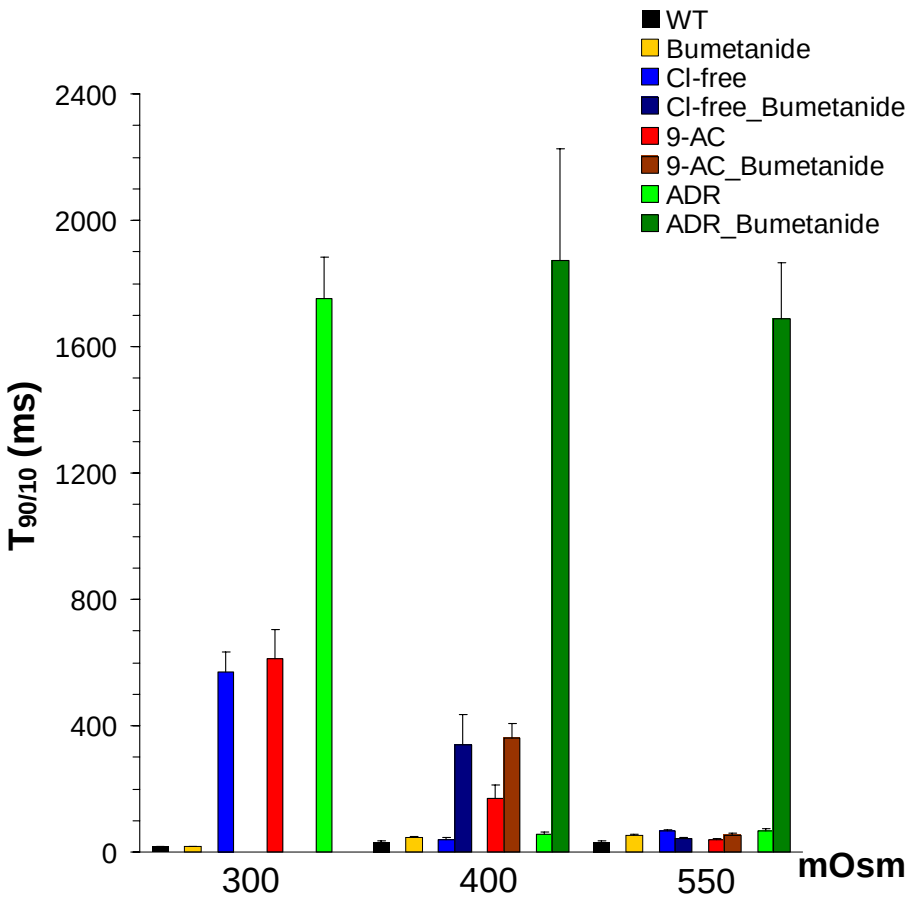


Figure 19: Antimyotonic effects of high osmolarity are partially antagonized by the specific NKCC1 inhibitor bumetanide.

Data represent mean values of 20 single twitches of WT (n=22), Cl^--free (n=7), 9-AC (n=8) and ADR (n=10) gastrocnemius muscle. Application of 100 µM bumetanide significantly reversed the antimyotonic effects.

3.1.5 Pharmacological investigations

In this part, several sets of experiments were performed using pharmacological substances influencing Na^+ and K^+ conductance. Substances were added into the bath solution as described in the method section and at the dosages specified in Table 3. The osmolarity of the final solutions was adjusted to 300 mOsm and pH was equilibrated to 7.4.

3.1.5.1 Substances influencing the $Na_v1.4$ channel

The $Na_v1.4$ channel blocker mexiletine is in clinical use for the treatment of myotonic syndromes. To test the in vitro effects of $Na_v1.4$ channel blockers, flecainide and mexiletine were applied to ADR muscle.

Administration of mexiletine to ADR muscle caused a reduction of relaxation times (Fig. 20). This effect was dose-dependent. Besides, the drug increased the twitch force in ADR muscle. The average twitch force was 31.3 $\pm$ 2.5 mN with 50 µM mexiletine vs. 21.1 $\pm$ 2.0 mN without mexiletine.

Similar to mexiletine effects, flecainide dose-dependently reduced the myotonic activity. The twitch force increased after flecainide application (33.3 $\pm$ 1.9 mN at 50 µM) as well. However, mexiletine seems to be more specific because it showed a stronger antimyotonic effect at low µM-concentrations.

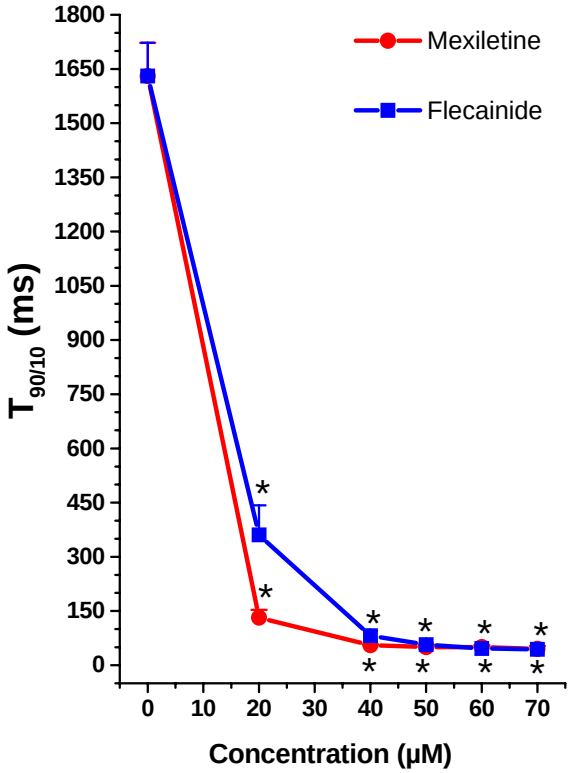


Figure 20: Dose-response curves of mexiletine and flecainide in ADR muscles.

ADR gastrocnemius samples were challenged with mexiletine (n=10) and flecainide (n=8). Symbols represent the mean values of 20 twitches. Mexiletine and flecainide had dose-dependent antimyotonic effects as shown by a decrease of $T_{90/10}$. * significant difference vs. 0 µM.

3.1.5.2 Substances influencing the KCNQ5 channel

As described in the introduction section, KCNQ5 channels are expressed on skeletal muscle enhancing membrane repolarization. A potent activator of KCNQ5 channels is retigabine and a specific inhibitor is XE991. The substances were tested on ADR-gastrocnemius muscle. The relaxation time of contractions after retigabine and XE991 administration was analysed. As shown in Fig. 21, retigabine dose-dependently reduced the relaxation deficit of ADR-myotonic contraction. Besides a decrease of $T_{90/10}$, administration of retigabine also alleviated the build-up of force in myotonic contractions. At 100 µM retigabine, the myotonic activity was abolished ($T_{90/10}$ = 61.0 ± 12.5 ms, force = 16.7 ± 2.4 mN).

In contrast to retigabine, XE991 (30 µM) application to ADR muscle (n=20) did not significantly alter the relaxation time and the twitch force. The average values of the $T_{90/10}$ and the twitch force compared to ADR muscle were 1538.2 ± 123.5 ms vs. 1630.5 ± 91.8 ms and 30.0 ± 3.7 mN vs. 25.63 ± 1.9 mN, respectively.

In summary, the results of the retigabine experiments support the hypothesis that an increase of gK^+ by activation of KCNQ5 enhances the warm-up phenomenon in myotonic muscle.

The next step was to confirm the animal data of retigabine on human muscle. A sample from human musculus vastus lateralis was dissected and experimental myotonia was pharmacologically induced by using 9-AC as described in the method section. A representative force registration of human muscle is displayed in Fig. 22. Again, retigabine alleviated the build-up of force and promoted the warm-up phenomenon in myotonic muscle.

Furthermore, a sample of a paramyotonia congenita patient was available. In this condition, the sodium channel inactivation is impaired. Likewise, retigabine decreased the relaxation time (Fig. 23). The data on human samples strongly confirmed that retigabine is an effective antimyotonic drug.

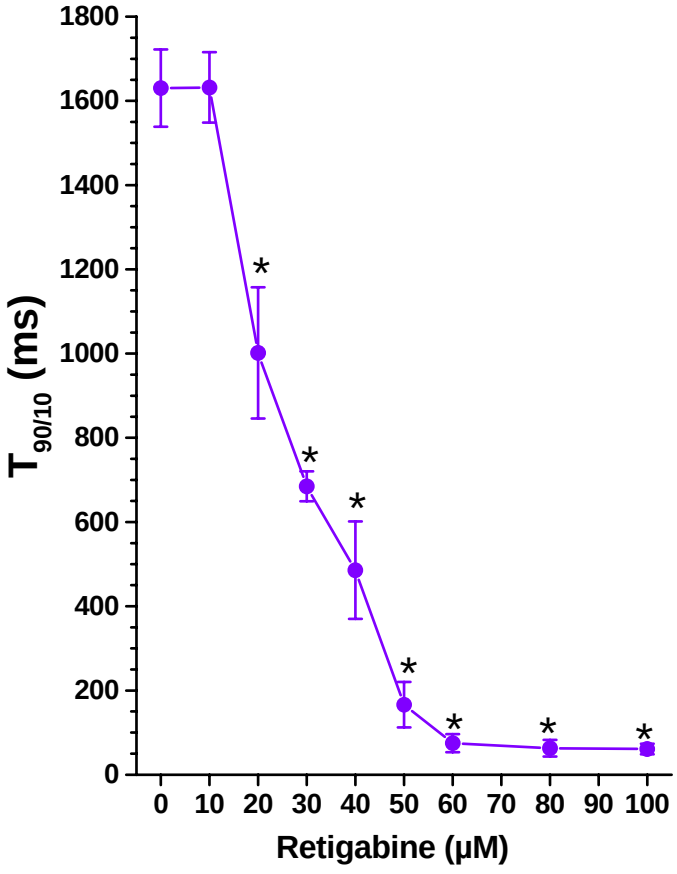


Figure 21: Dose-response curve of retigabine in ADR muscles.

Data were obtained from the mean values of 20 single twitches. Application of retigabine to ADR muscle (n=10) dose-dependently decrease the relaxation time ($T_{90/10}$). * significant difference vs. 0 µM

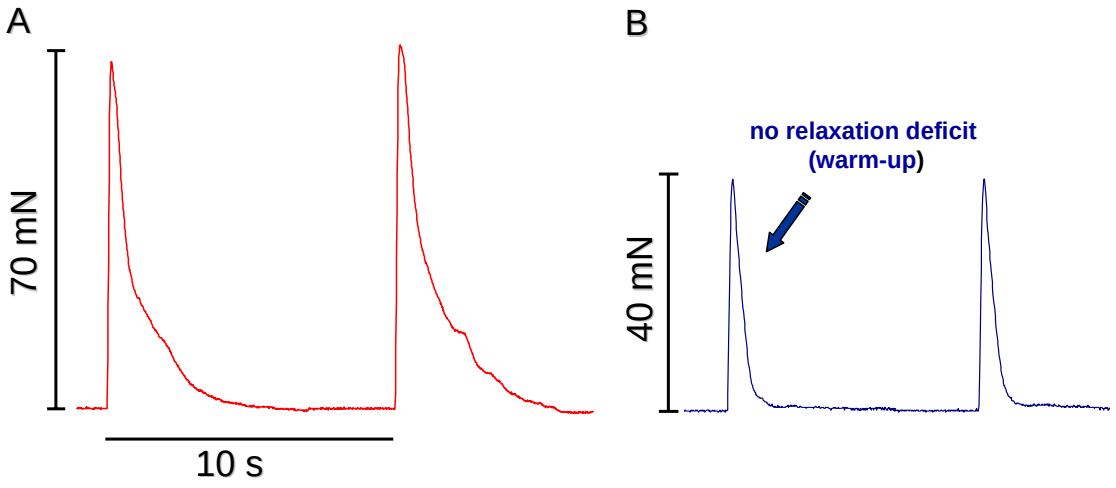


Figure 22: Effect of retigabine in human samples.

Myotonia was pharmacologically induced in the muscle strip of a healthy human donor by 9-AC. The strip was electrically stimulated with 1 ms pulse at 0.1 Hz. A) Myotonic activity shows a build-up of force and a slowed relaxation. B) Twitch responses were observed after 20 min administration of 100 µM retigabine.

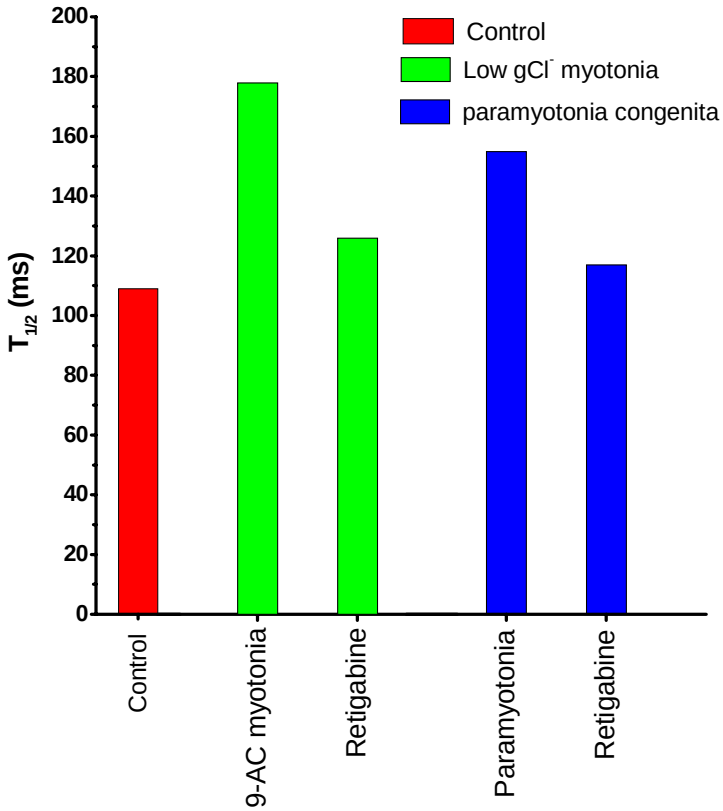


Figure 23: Effect of retigabine in human musculus vastus lateralis.

Force measurements were performed on the muscle strip of 9-AC induced low gCl^- myotonia and in a sample of a paramyotonia congenita patient. The relaxation time ($T_{1/2}$) of twitch contraction was recorded before and after 100 µM retigabine application. Strong myotonia induced by 100 µM 9-AC reflects a high relaxation time ($T_{1/2}$). Administration of the KCNQ5 activator retigabine to myotonic muscle reduced $T_{1/2}$.

3.1.5.3 Substances influencing the BK channel

The BK channels are dominantly localized on the T-tubular membrane. This channel is important for repolarizing phase of an AP. An interesting finding in BK knock out mice is myotonia could not emerge without BK channels (Sartorius et al. 2006). The insight led to an investigation of the functional properties of BK channels concerning the warm-up phenomenon in myotonia. BK channels were activated by NS1608 and blocked by paxilline. The substance was tested in control and myotonic muscle.

Fig. 24 demonstrates the results of pharmacologically 9-AC induced myotonia in WT-soleus and -EDL muscle. The interesting finding is NS1608 had the opposite effect to paxilline on the onset of myotonia. Application of NS1608 to 9-AC and ADR muscle (Fig. 25) aggravated the onset of myotonic activity by prolongation of $T_{90/10}$ in some first twitches. Additionally, NS 1608 led to a more rapid onset of warm-up compared to myotonic muscle.

Application of paxilline to control muscles (WT-soleus, n=4) itself caused a slight increase of $T_{90/10}$. This effect will be discussed in the discussion section (see 4.4). Surprisingly, in 9-AC soleus and EDL, paxilline significantly shortened the relaxation time of contractions. Likewise, challenging ADR muscle with paxilline led to a reduction of $T_{90/10}$. In addition, paxilline reduced the twitch force slightly in all myotonic muscles (data not shown). It can be concluded that an efflux of K^+ through BK channels during the muscle activity involves on the onset of myotonic stiffness.

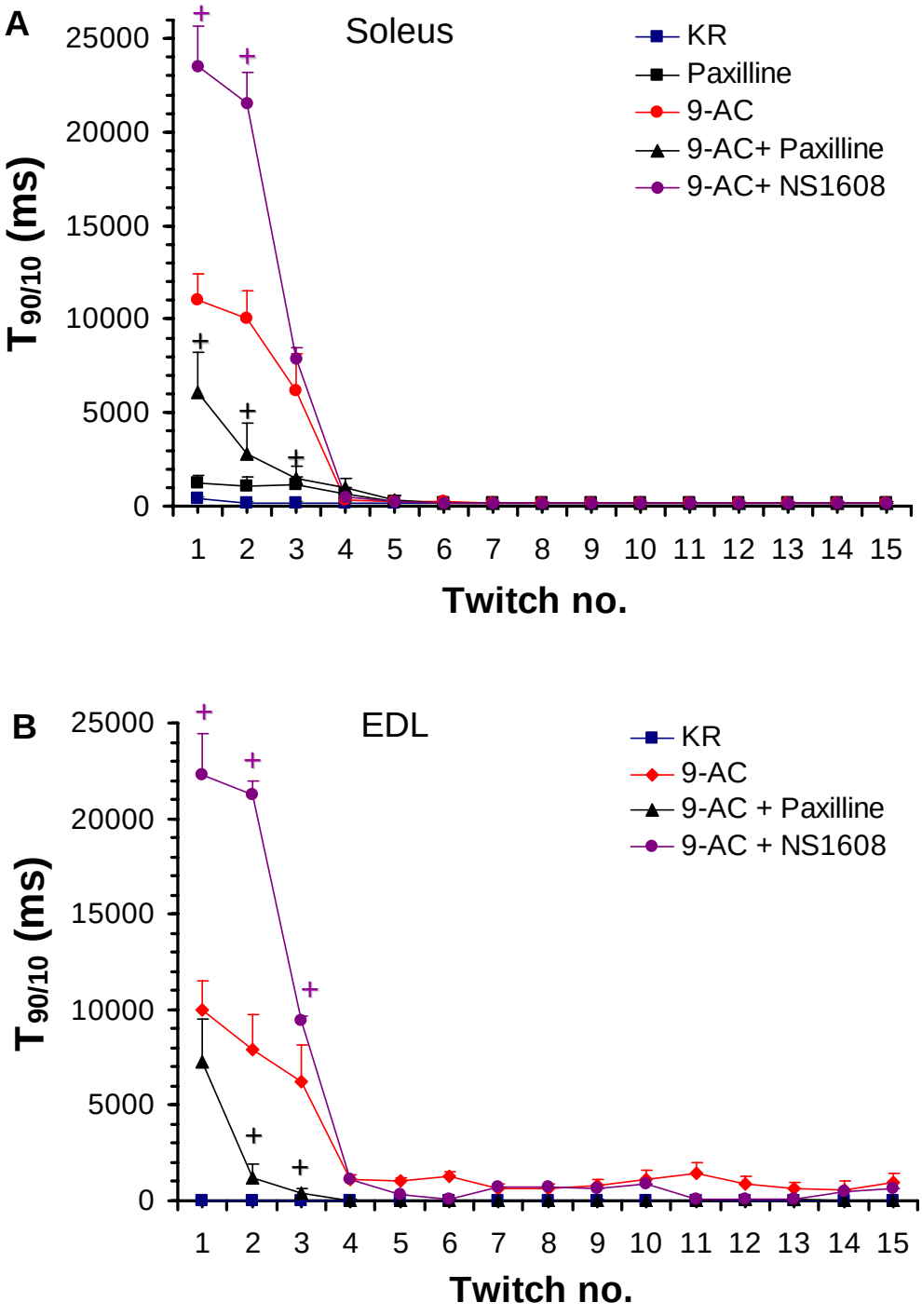


Figure 24: Effect of paxilline and NS1608 in 9-AC induced low gCl^- myotonia.

The effect of the BK channel blocker paxilline and the BK channel activator NS1608 on 9-AC induced low gCl^- myotonia was investigated. Twitch responses were recorded on 6 bundles of soleus (A) and EDL (B) isolated muscle after application of paxilline (20 µM) and NS 1608 (20 µM). Paxilline caused a decrease in $T_{90/10}$ in 9-AC myotonic muscle whereas NS1608 had opposite effects. Data displayed the $T_{90/10}$ from the first 15 twitches in WT and 9-AC myotonic muscles. + significant difference vs. 9-AC.

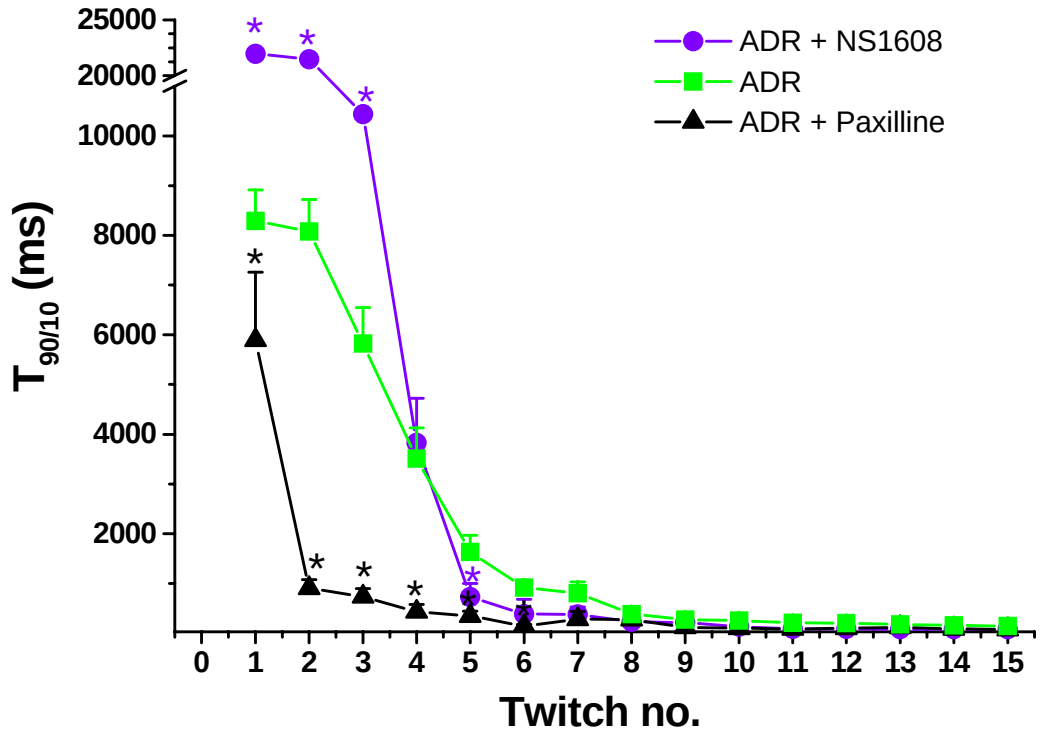


Figure 25: Effect of paxilline and NS1608 in ADR muscles.

The effect of paxilline and NS1608 on ADR gastrocnemius muscle was investigated. Blocking BK channels with paxilline (20 µM, n=11) caused a decrease in $T_{90/10}$ whereas activating the channels with NS1608 (20 µM, n=7) had opposite effects in ADR myotonic muscle (ADR=30). Data displayed $T_{90/10}$ from the first 15 twitches in ADR muscles. * significant difference vs. ADR.

To confirm the results of BK channel modifiers on animal muscle, NS1608 and paxilline were also tested on human tissue obtained from healthy donors. Isopimaric acid, another BK channel activator, was examined as well.

All drugs did not significantly change contraction and relaxation parameters in control conditions. Challenging the control strips with isopimaric acid (10 µM) slightly increased the twitch force (52.5 $\pm$ 4.8 mN, n=5) when compared to the control (44.5 $\pm$ 3.3 mN). However, in pharmacologically induced low gCl^- myotonia, paxilline and NS1608 significantly altered the myotonic activity as shown in Fig. 26. Paxilline significantly reduced the relaxation time and the twitch force in human samples. In other words, blocking the BK channel prevents the onset of myotonia.

Again, NS1608 had the opposite effect to paxilline by aggravating the myotonic activity. These findings confirm the data obtained from the murine myotonia models.

In summary, the onset of myotonia could be prevented by inhibition of BK channels and aggravated by activation. However, activation of BK channels accelerated the onset of warm-up phenomenon.

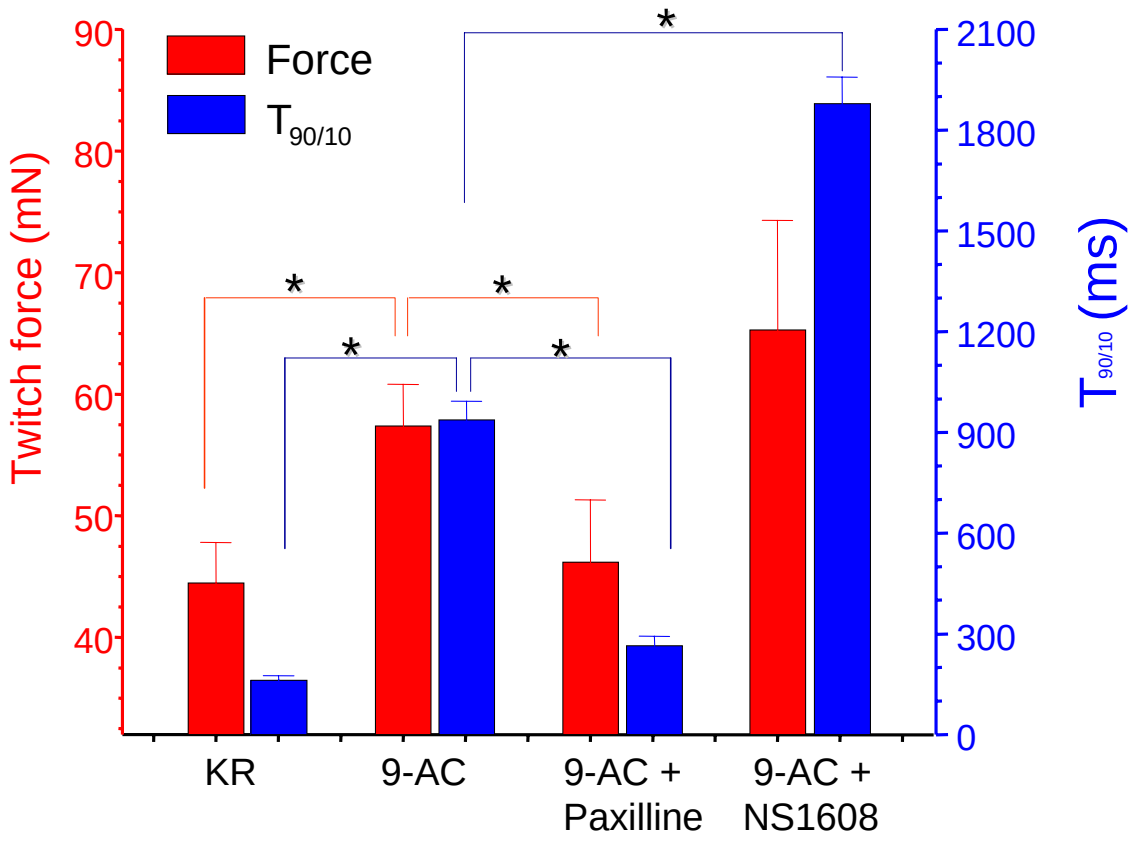


Figure 26: Effect of paxilline and NS1608 in 9-AC induced low gCl^- myotonia in human samples.

Data were obtained from the first twitch contraction in vastus lateralis muscle obtained from healthy donors (n=8). Low gCl^- myotonia was pharmacologically induced by 100 µM 9-AC in 4.5 mM $[K^+]_o$ KR. The BK^+ channel blocker paxilline (n=4) and the BK^+ channel opener NS 1608 (n=4) were applied to 9-AC myotonic muscle at the concentration of 20 µM. * significant difference vs. 9-AC.

3.1.5.4 Caffeine and halothane contracture

Excitation contraction coupling (EC-coupling) is modulated by Ca^{2+} release from SR through ryanodine receptors type 1 (RyR1). To investigate the effects of RyR1 activators on ADR muscle, isolated gastrocnemius bundles from ADR and WT mice were challenged with the potent RyR1 activators caffeine and halothane.

Fig. 27 shows the result as a mN-shift of baseline after challenging the samples with increasing concentrations of caffeine respectively halothane. The drugs did not evoke a contracture at the used concentrations in the low mM range. There was a drop of force in the ADR samples, which was not statistically significant. In addition, there was no influence on the relaxation parameters (data not shown).

The effects of RyR1 activators caffeine and halothane were also investigated on a muscle specimen obtained from a human donor suffering from paramyotonia congenita. Again, there were no pathologic contractures in the caffeine / halothane test. The relaxation parameters were not affected.

These results corroborate the view that Ca^{2+} release from RyR1 is not causative for the initiation of myotonic stiffness.

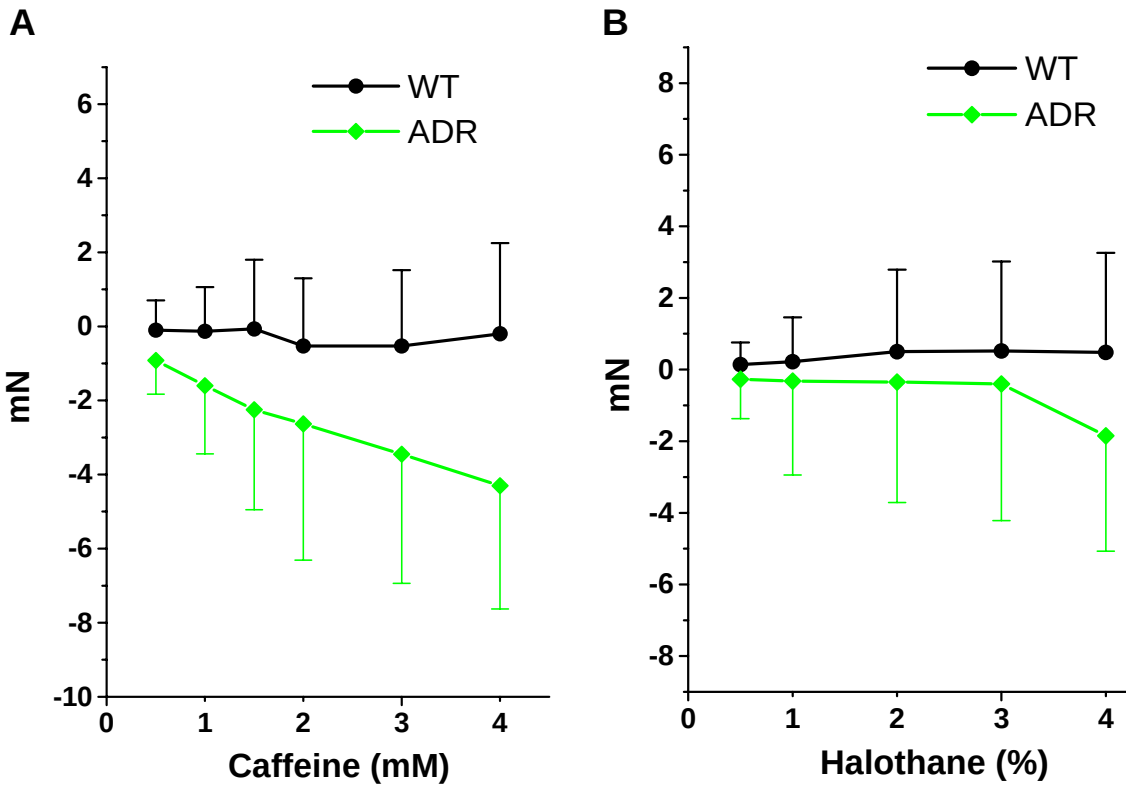


Figure 27: Caffeine and halothane - sensitivity of myotonic muscle.

Baseline tension was recorded in ADR (n=4) and WT (n=4) gastrocnemius muscle. Caffeine (0.5 - 4 mM, A) respectively halothane (0.5 - 4 %v/v, B) was applied to the tissue bath (4.5 mM $[K^+]_o$ KR) in increasing concentrations.

3.2 INTERNAL MICROELECTRODE MEASUREMENTES

3.2.1 Resting membrane potential measurements

This study monitored the instability of the RMP in WT and ADR mice under various conditions of $[K^+]_o$. In this step, hemidiaphragm was used because of the technical reason that diaphragm is a flat muscle and it is easy to impale.

In physiological KR-solution (3.5 mM $[K^+]_o$, 290 mOsm), ADR tissue revealed a less negative RMP (-75.80 $\pm$ 2.59 mV, n=9) than control (-79.55 $\pm$ 2.23 mV, n=9). This shift of roughly 4 mV reached the level of statistical significance.

3.2.2 Myotonic burst in ADR muscle

The electrophysiological correlate of myotonia is involuntary repetitive firing of muscle fiber AP's, the so-called myotonic burst. EMG needle insertion into the resting muscle of myotonic patients itself elicits the typical myotonic bursts. Under physiological conditions, impalement with the glass microelectrodes evoked a series of spontaneous AP in the ADR muscle fibers (Fig. 28). The effect was not seen in WT muscle fibers. This is the in vitro correlate of the burst pattern seen in EMG investigations.

The amplitude of these potential bursts did not vary significantly along the fiber segments. In physiological KR solutions, the bursts were present in 85% (range from 70-100%) of 220 impaled muscle fibers from ten different ADR animals. Withdrawal of the electrode caused also one or a few small AP bursts.

Adding the Na^+ channel blocker tetrodotoxin (TTX, 500 nM) to the bath solution could abolish repetitive bursts.

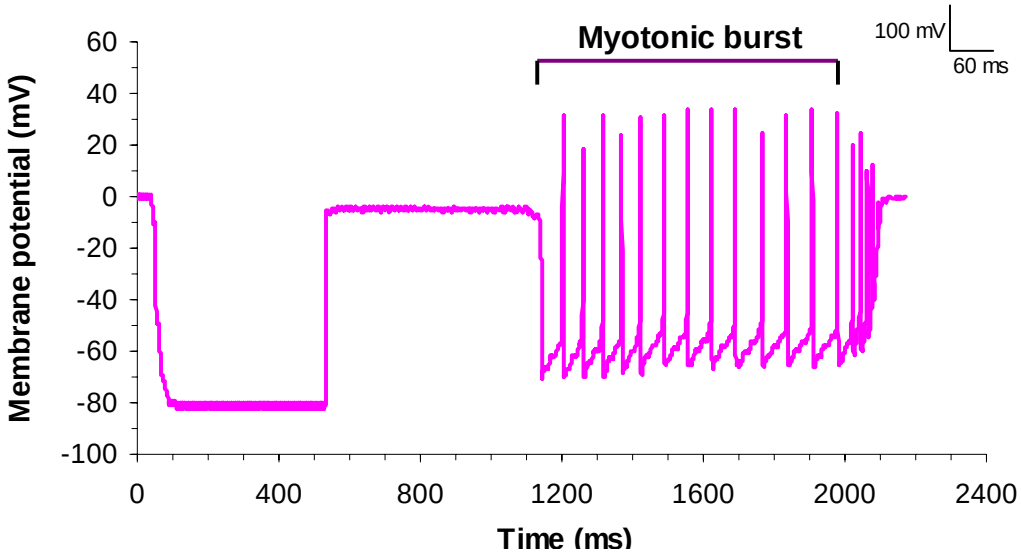


Figure 28: RMP in a hemidiaphragm preparation of ADR mice.

A sample recording of the RMP in an ADR hemidiaphragm after 15 min incubation in 3.5 mM $[K^+]_o$ KR solution at room temperature. Insertion of the microelectrode into the second fiber of the isolated muscle bundle elicits a myotonic burst.

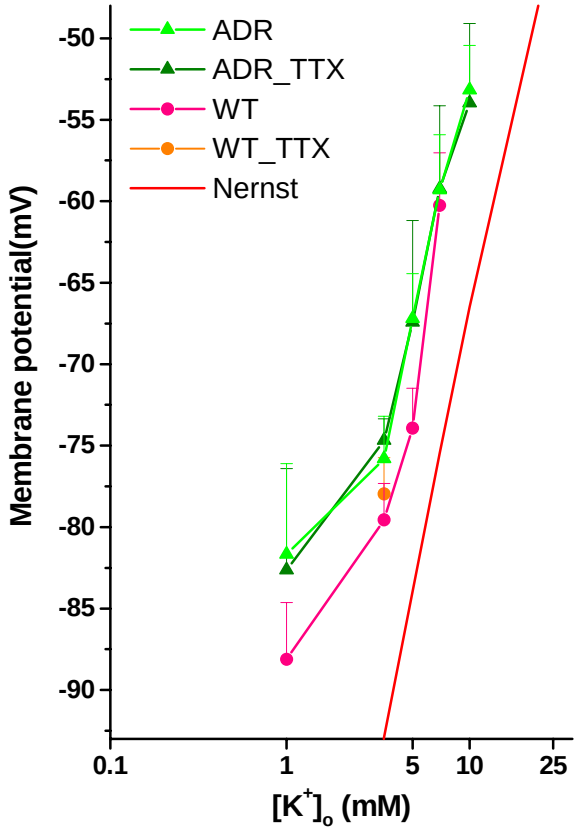


Figure 29: K^+ effects on RMP.

Application of 500 nM TTX to ADR hemidiaphragm (ADR_TTX) and WT (WT_TTX) in 1, 3.5, 5, 7 and 10 mM $[K^+]_o$ KR caused no statistically significant changes of RMP. The red curve illustrates the theoretical K^+ equilibrium potential calculated by Nernst's equation. ADR_average and WT_average are the mean RMP value of before and after TTX application.

3.2.3 Influence of $[K^+]_o$ on RMP and myotonic burst

A potential mechanism of the antimyotonic effect at high $[K^+]_o$ is a membrane depolarization of myotonic muscle fibers that might terminate the myotonic burst. To examine the hypothesis the RMP and myotonic bursts were recorded in ADR tissue at various $[K^+]_o$.

To suppress the generation of spontaneous AP's, most of the RMP measurements were made in the presence of 500 nM TTX in the bath solution. In physiological KR solution, TTX caused a shift of +1.11 $\pm$ 1.34 mV in ADR samples (n=14) and +1.98 $\pm$ 2.23 mV in WT (n=14). However, these changes were not statistically significant (Fig. 29).

Fig. 30 clearly shows that raising $[K^+]_o$ dose-dependently depolarized the RMP of WT- and ADR-muscle fibers. Exposure to high $[K^+]_o$ caused a reduction of myotonic bursts. The myotonic bursts in ADR were plotted in Fig. 30 as percentage of fibers at the various $[K^+]_o$. When $[K^+]_o$ was raised to 5 mM and 7 mM, the rate of myotonic bursts decreased significantly. In addition, myotonic bursts with lower frequency were observed some fibers at 5 and 7 mM $[K^+]_o$ (data not shown). At 10 mM $[K^+]_o$, the RMP was depolarized to 53.43 $\pm$ 2.42 mV and the myotonic bursts were absent.

In summary, an increase in $[K^+]_o$ led to a sustained membrane depolarization going along with a reduction of myotonic bursts in ADR mice.

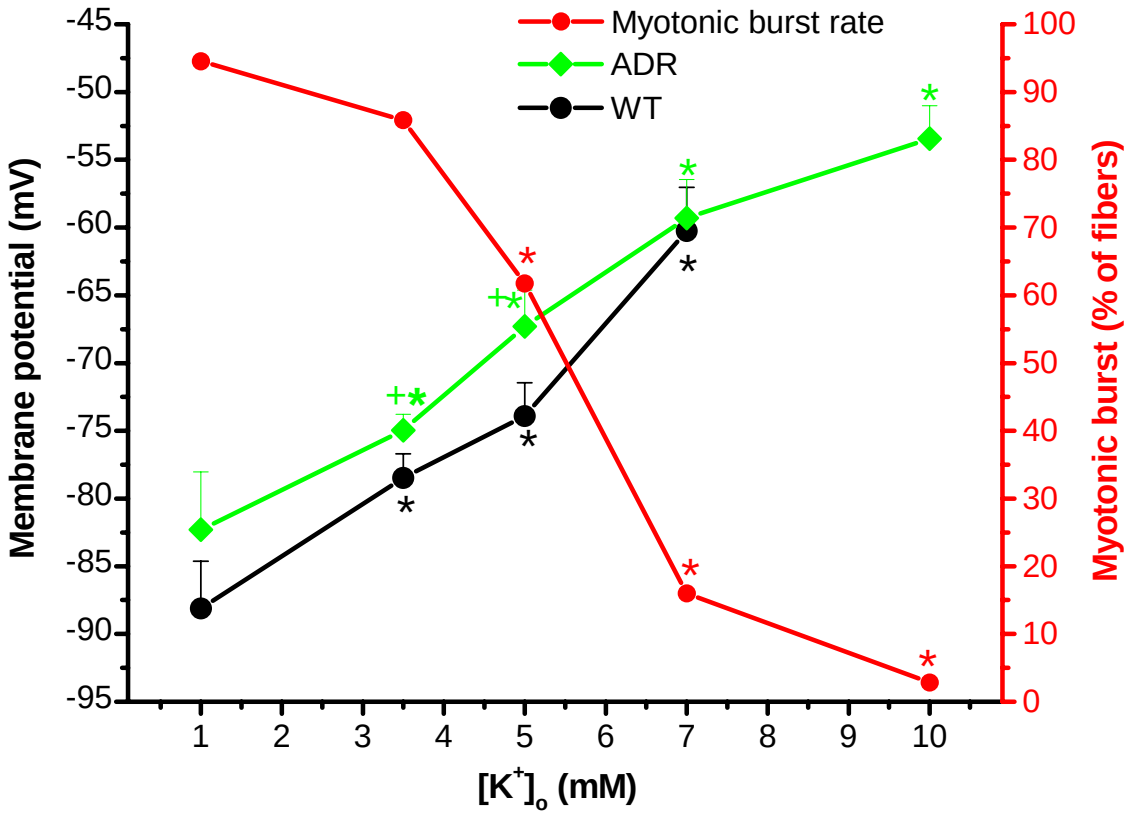


Figure 30: Influence of $[K^+]_o$ on RMP and myotonic bursts.

Increasing $[K^+]_o$ enhanced the depolarization of RMP in WT and ADR hemidiaphragm preparations as well as a reduction of myotonic bursts in ADR preparations. The mean RMP were obtained from 22 ADR and 16 WT at 3.5 mM $[K^+]_o$ and 8 animals at other $[K^+]_o$ (20-30 fibers per animal). The myotonic bursts are shown as the percentage of muscle fibers. The rate of myotonic bursts were obtained from 10 different ADR animals at 3.5 mM $[K^+]_o$, and 6 ADR at other $[K^+]_o$ (20-25 fibers per animal). + significant difference vs. WT. * significant difference vs. 1 mM $[K^+]_o$.

3.2.4 Influence of osmolarity on RMP and myotonic burst

The surprising finding that high osmolarity had antimyotonic effects and facilitated the warm-up phenomenon (see 2.1.4) led to RMP-investigations under hyperosmotic conditions. The RMP and myotonic burst rate were recorded in ADR and WT (Fig. 31 and Fig. 32).

Interestingly, elevating osmolarity caused a significant depolarization of the RMP in WT and ADR mice tissue (Fig. 32). The membrane depolarization of WT was larger than that of ADR, but the difference did not reach statistical significance. Similar to high $[K^+]_o$, hyperosmolarity significantly reduced the rate of myotonic bursts in ADR mice (Fig. 31). Additionally, AP bursts under hypertonic conditions had a lower frequency than at physiological conditions (data not shown).

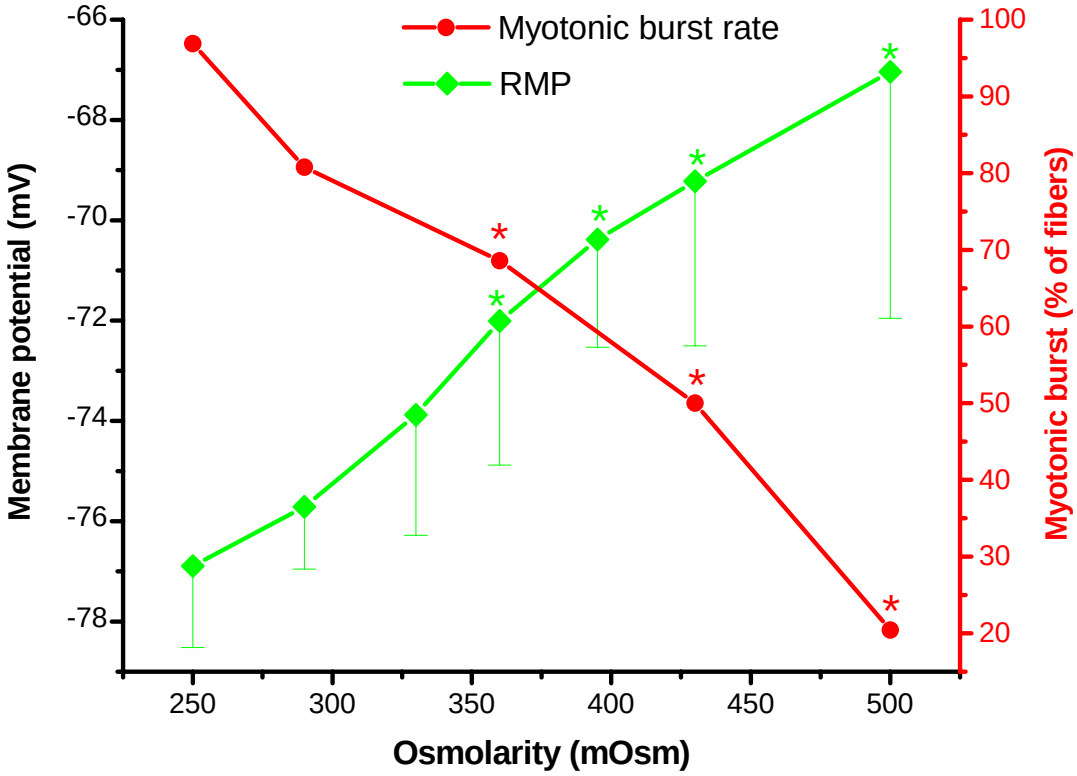


Figure 31: Influence of osmolarity on RMP and myotonic bursts.

The RMP was obtained from ADR hemidiaphragm as mean values of 25-30 fibers per animal at 250 mOsm (n=13), 290 mOsm (n=17), 330 mOsm (n=10), 360 mOsm (n=11), 395 mOsm (n=14) and 430, 500 mOsm (n=6). The myotonic bursts are illustrated as the percentage of muscle fibers from 75 fibers at 250 mOsm, from 210 fibers at 290 mOsm, from 75 fibers at 360 mOsm and from 70 fibers at 430 mOsm and 500 mOsm. * significant difference vs. 250 mOsm.

3.2.5 NKCC1 inhibition under hyperosmotic conditions

As described above, membrane depolarization and a reduction of the myotonic burst rate was observed under hyperosmotic conditions. To confirm the hypothesis that NKCC1 plays an important role on the antimyotonic effect (see 3.1.4.3), the RMP was recorded in WT and ADR hemidiaphragm after 15 min exposure to bumetanide under hyperosmotic conditions.

Fig. 31 displays the depolarization of the resting membrane under hyperosmotic conditions in WT and ADR fibers. It is an interesting finding that in osmotic stress ADR fiber was depolarized to a lesser extent than WT samples. The reason might be found in a compensatory alteration of the protein expression pattern in ADR muscle. After exposure to bumetanide, the membrane depolarizations were significantly suppressed at 350, 395,

430 and 500 mOsm. These results indicate that the NKCC1 activation plays an important role on the membrane depolarization in hypertonicity.

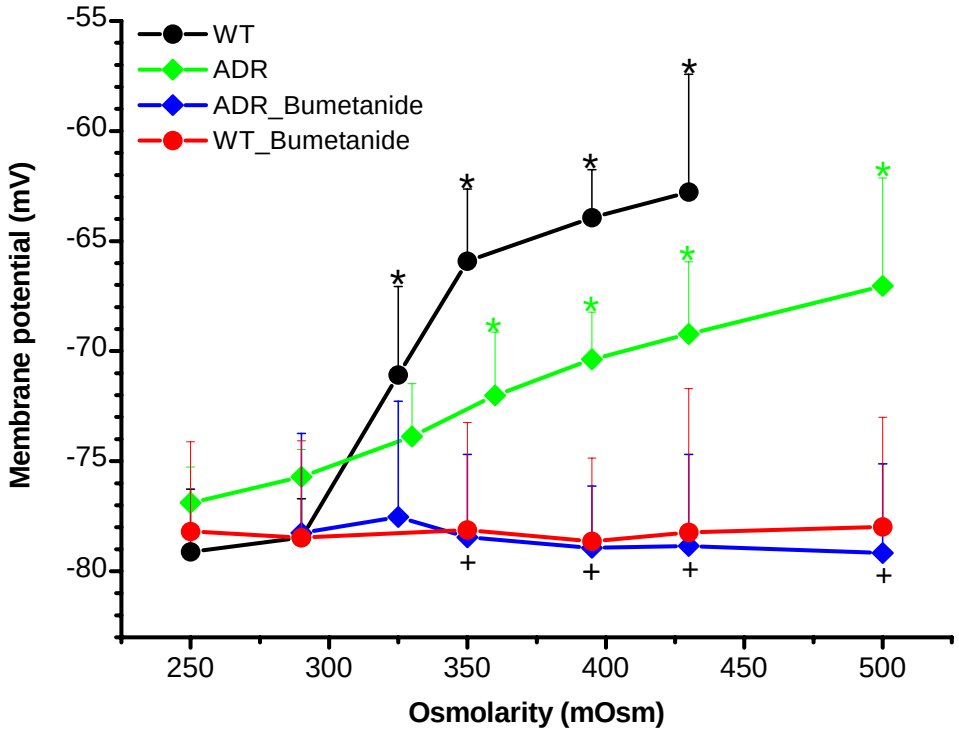


Figure 32: Inhibition of membrane depolarization in hyperosmotic solution by bumetanide.

Increasing osmolarity caused membrane depolarization in WT and ADR hemidiaphragm. Addition of 50 µM bumetanide to WT-muscle (WT_bumetanide, n=5) and ADR-muscle (ADR_bumetanide, n=8) significantly prevented the membrane depolarization, particularly, under hyperosmotic conditions. + significant difference vs. WT and ADR. * significant difference vs. 290 mOsm.

3.2.6 Full warm-up frequency and high $[K^+]_o$

As an earlier finding, prolonged tetanic stimulation of ADR diaphragm led to the warm-up phenomenon (see Table 5). At 100 Hz myotonia was absent. To investigate the electrical response of myotonic fibers at the warm-up state, the RMP and the rate of myotonic bursts were observed after the double tetanus stimulation at 100 Hz at a 1 min interval.

In physiological solution (4 mM $[K^+]_o$), the rate of myotonic bursts of ADR tissue was significantly decreased in ADR-warmed-up tissue compared to ADR-control tissue (Fig. 33A). This indicates that the myotonic bursts were suppressed by the double tetanus stimulation with the full warm-up frequency.

In order to confirm the potential role of high $[K^+]_o$ on the warm-up phenomenon, the rate of myotonic bursts was recorded in low (1 mM) and high (7 mM) $[K^+]_o$. Also, the RMP was measured. Fig. 33A shows that the double tetanus stimuli enhanced a reduction of the myotonic burst rate. The figure helps to look at the potassium independent warm-up effects. At a clamp $[K^+]_o$ of 4 mM the burst rate was reduced by a factor of roughly 2.

Increasing $[K^+]_o$ significantly depolarized the RMP of ADR warmed-up tissue. This effect confirms the previous finding of membrane depolarization following exposure to high $[K^+]_o$ (see Fig. 33B). ADR-warmed-up tissue revealed a more negative RMP than in ADR-control tissue. The RMP curve had a right shift to the theoretical K^+ equilibrium potential (E_K) calculated by Nernst's equation. The RMP shift reached the level of statistical significance only at 7 mM $[K^+]_o$.

These results indicate that the warm-up phenomenon has a $[K^+]_o$ independent component. There is no doubt that high $[K^+]$ enhances the phenomenon. However, at constant $[K^+]_o$ the warm-up still occurs and goes along with a slight hyperpolarization.

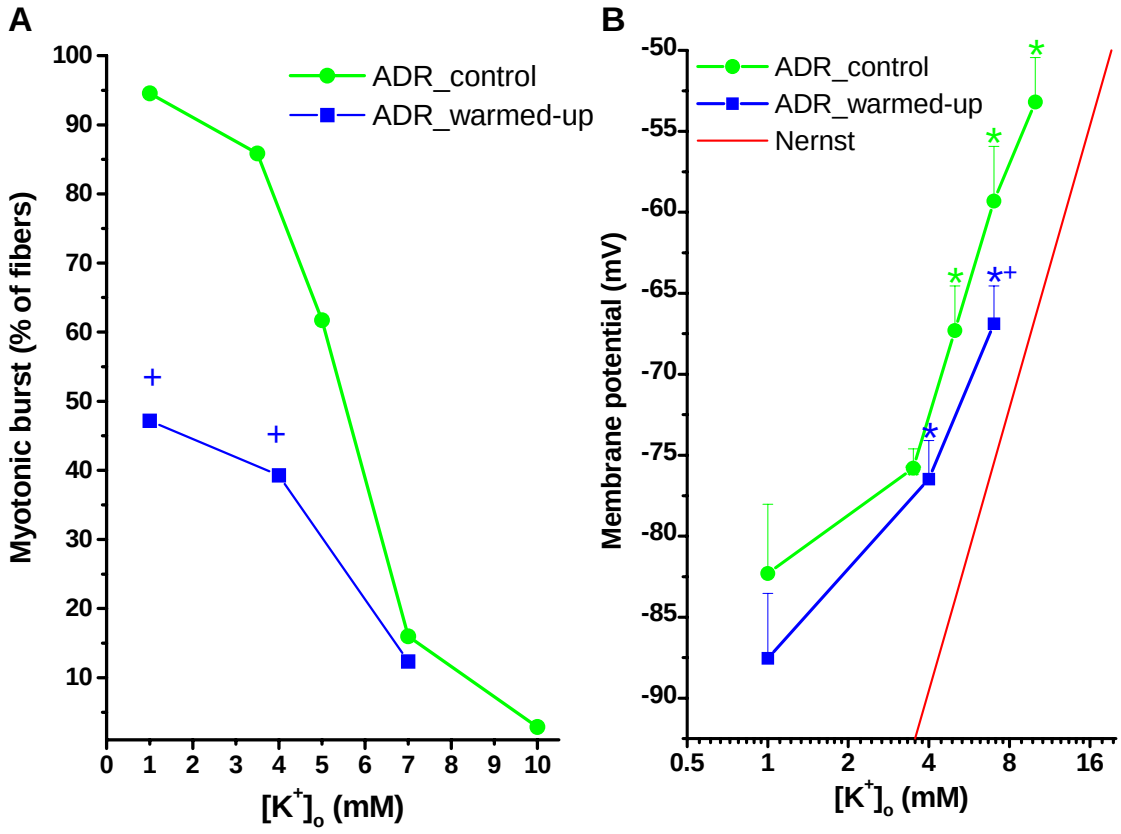


Figure 33: Effects of tetanic stimulation with full warm-up frequency and $[K^+]_o$ on myotonic bursts and RMP.

The rate of myotonic bursts (A) and the RMP (B) were obtained from ADR hemidiaphragm after 2 consecutive tetanic contractions at the frequency of 100 Hz (ADR_warmed-up, n=8), ADR hemidiaphragm before tetanic stimulations (ADR_control, n=8). Raising $[K^+]_o$ enhanced significantly a reduction of myotonic bursts (A) and membrane depolarization (B). The red curve illustrates the theoretical K^+ equilibrium potential calculated by Nernst's equation. * significant difference vs. 1 mM $[K^+]_o$. + significant difference vs. ADR_control.

4 DISCUSSION

4.1 RESTING CONDITIONS IN MYOTONIC MUSCLE

Myotonic AP-bursts and the typical myotonic contraction pattern were clearly observed in this study in several models for low gCl^- myotonia. In low gCl^- myotonic muscle, mean gCl^- is decreased to at least 20% of normal or less and mean gK^+ is about 2 times higher than normal (Bryant 1982, Kwiecinski et al. 1988, Lehmann-Horn et al. 2007). According to the Nernst's equation, the reversal potential for Cl^- is roughly 5 mV less negative than for K^+. The present study demonstrated that the RMP in myotonic ADR muscle yielded a shift of 3.9 $\pm$ 2.2 mV to less negative values in comparison to control fibers at resting conditions. The slight membrane depolarization of ADR fibers was also shown in the presence of TTX, which suppresses the spontaneous generation of AP's. Therefore, these data strongly confirm literature findings, that the depolarization is a true phenomenon of myotonia rather than an artifact due to spontaneous activity (Kurihara 1977, Barchi 1978). In this context it is interesting to state that myotonic goat have a serum K^+ level which is 0.5 mM above the control group (Bryant et al. 1968).

4.2 MYOTONIC STIFFNESS

The clinical syndrome in low gCl^- myotonia patients is characterized by a relaxation deficit (i.e. stiffness) after forceful voluntary contractions followed by transient weakness. Excess of K^+ in the T-system is the most probable cause for the myotonic phenotype (Cannon et al. 1993, Wallinga et al. 1999). Activation of gross muscle during exercise leads to a rise in serum K^+ levels up to 10 mM (Juel et al. 2000, Pedersen et al. 2005). If K^+ is elevated during muscle activity and gCl^- is low, the membrane does not readily repolarize beyond the K^+ equilibrium potential (E_K). In turn, this leads to an accumulation of $[K^+]_o$. The T-system is particularly vulnerable to changes in $[K^+]_o$ and to gK^+ because of its extremely large surface-to-volume ratio. Comprising only a small portion of extracellular volume (i.e. 0.32% of the fiber volume) $[K^+]_o$ can rise significantly during muscle activation (Mobley and Eisenberg 1975). According to the model of Wallinga et al. (1999) one single AP leads to a rise of 0.4 mM in T-tubular $[K^+]$. Clausen (2003) calculated for non-myotonic muscle that stimulation of gross muscle at 40 Hz increases the T-tubular $[K^+]$ to ~15 mM in a time-scale of seconds, sufficient to block AP propagation. This implies that in the present

study the T-tubular [K^+] at full warm-up-frequency might have risen above 10 mM. Therefore, the K^+ gradients across the T-tubular membrane are far different from estimation by serum K^+ levels.

The K^+-accumulation in the T-tubular lumen depolarizes the membrane sufficiently to initiate self-sustaining AP's (i.e. myotonic bursts). The spontaneous AP's activate the dihydropyridine receptor (DHPR), which is the key for excitation-contraction coupling. The excitation-contraction coupling in mammalian muscle fibers involves AP propagation in transverse as well as in longitudinal direction (Posterino et al. 2000). The resulting prolonged muscle contraction (i.e. myotonic stiffness) was shown in this and other studies for single twitch as well as for tetanic contraction (Adrian and Bryant 1974, Barchi 2001, Van Lunteren et al. 2007, this study). Myotonic T-tubules presumably have an electrochemical K^+ equilibrium far from the physiological conditions of the sarcolemma. It can be speculated that the AP-repolarization via K^+ efflux is additionally decelerated if there is a build-up of K^+ inside the T-tubular compartment.

The autonomous T-tubular excitation is not able to generate a recurrent AP on the surface membrane. The reason for this unidirectional transmission has been attributed to the anatomical properties of the T-tubular microstructure (Lamb 2005). The T-system constitutes a functional compartment and K^+ diffusion to and from outer space is reduced by a factor of 5 in comparison to the value in free solution (~85 $\mu m^2/s$) (Swift et al. 2006). Ion equilibration of the T-system with the outer interstitial space will take several minutes. The long incubation experiments (>20 min) in this study show that there is a time-dependent K^+ effect on twitch upstroke and relaxation parameters. It has been shown that an increase in extracellular Ca^{+2} or Mg^{+2} depresses the AP frequency or hampers the AP generation (Orchardson 1978). Most likely this is caused by a cloud of bivalent cations forming an electrochemical shield which surrounds each myofiber. These ions are wrapped by a jacket of water dipoles corresponding to their electronegativity ($Ca^{+2} > Mg^{+2}$) which spans the narrow T-tubular opening (Aidley and Stanfield 1997). Na^+ diffusion into the T-system is essential for T-tubular excitability, and is hampered by the shield as well as a high T-tubular K^+ concentration.

4.3 TRANSIENT WEAKNESS

The myotonic burst and muscle excitation will further depolarize the T-system. Surface EMG registrations revealed less and slower sarcolemmal AP bursts during the transient

weakness (Van Beekvelt et al. 2006). At potentials -59.7 ± 4.5 mV the $Na_v1.4$ channels inactivate. This inactivation state renders the affected membrane hypoexcitable (Lehmann-Horn et al. 1987, Cannon 1993, Lehmann-Horn and Jurkat-Rott 2001). Although the AP propagation is hindered and AP-frequency is lowered, excitation-contraction coupling takes place and is still effective enough to sustain a measurable force (Van Beekvelt et al. 2006). In non-myotonic muscle with experimental AP-propagation block, there is even a force increase with reduced stimulation frequency (Cairns et al. 2003).

Most likely the surface membrane is still excitable whereas full inactivation of the $Na_v1.4$ channels is confined to some T-tubules. At least for exercise-induced fatigue a loss of sarcolemmal excitability has been excluded (Paterson 1996). The inactivation of $Na_v1.4$ channels leads to an AP-propagation block into some T-tubules. Also the longitudinal inter T-tubular communication is impaired, which in physiological conditions serves as an important safety mechanism for reducing conduction failure (Posterino et al. 2000).

Previously, Adrian and Bryant (1974) described that a depolarization of 10-30 mV forces enough $Na_v1.4$ channels into the inactivated state. This also was shown for myotonic muscle (Fanning and Macdermott 1997, Lehmann-Horn and Jurkat-Rott 1999). The findings of this dissertation showed that the myotonic burst rate was suppressed more than 90% at a membrane potential of -59.3 ± 2.8 mV (i.e. depolarization of 15.6 ± 2.1 mV). In other words T-tubular $[K^+]_o$ might have risen to more than 7 mM in this situation (see Fig. 30).

DHPR-inactivation takes several seconds and it is much slower than $Na_v1.4$ channel kinetics, which has a time-constant for fast inactivation of about 3 ms at the holding potential of -50 mV (Lehmann-Horn and Jurkat-Rott 2001). This mechanism also might contribute to the time-course of stiffness followed by transient weakness. In patients, the onset of transient weakness usually occurs within 5 s after the start of exercise. In this study, a marked transient weakness was observed in ADR isolated diaphragm within 1-3 s after initiation of tetanus-frequency stimulation with 50 Hz. The transient weakness lasted for approximately 4-6 s corresponding to the in vivo data (Van Beekvelt et al. 2006, this study). Therefore, a temporarily hypoexcitable T-tubular membrane induced by the above described propagation block can explain the transient weakness.

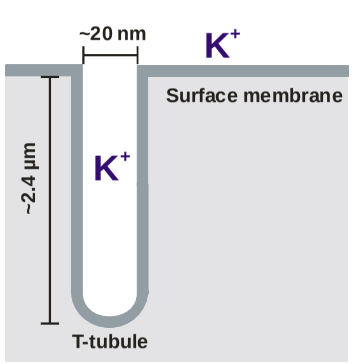
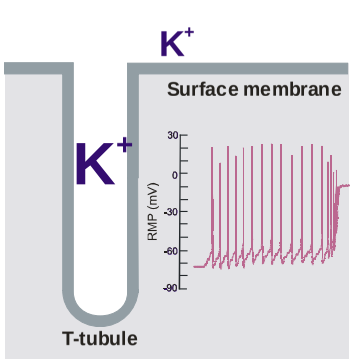
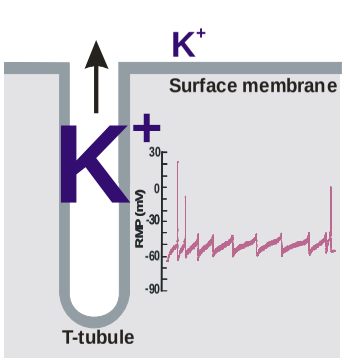
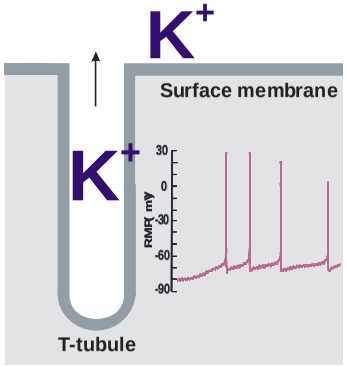
4.4 WARM-UP PHENOMENON

The end of the transient weakness and the onset of the warm up phenomenon are coincident. There is some overlap as well. The warm-up state wears off after approximately 5-8 minutes unless the muscle tissue is continuously activated (Birnberger and Klepzig 1979). Repetitive contractions with either twitch (0.1 Hz) or high-frequency tetani (see Table 5) alleviated the relaxation deficit as well as the transient weakness (i.e. the warm-up phenomenon). The present study shows variations of the onset of the warm-up for the different models of myotonia. ADR-muscle showed the most severe phenotype in terms of a late onset. Pharmacologically induced myotonia has a less severe phenotype and a more rapid onset of the warm-up although gCl^- is fully inhibited. This corroborates the view that compensatory changes in protein expression occur in low gCl^- myotonia during muscle development. Indeed, it has been shown that gK^+ is upregulated by a factor of 2 in myotonic muscle (Bryant 1982, Kwiecinski et al. 1988). On one hand increased gK^+ can compensate the lack of gCl^- to some extent, on the other hand it might worsen T-tubular K^+ accumulation (i.e. more severe phenotype, see ADR mice).

The warm-up phenomenon reflects an enhanced stabilization of the membrane excitability. Table 6 shows a hypothetical model for the factors influencing the T-tubular K^+-gradient and the potential contribution of various ion channels to the myotonic phenotype. The warm-up most likely is based on a combination of a reduced myoplasmic K^+ efflux, an enhanced clearance (K^+ effusion to outer space, K^+ reuptake) and/or activation of Cl^- transporting proteins.

Table 6: T-tubular K^+ kinetics.

e estimated value in the T-system**.** + indicates a probable contribution to the regulation of the T-tubular K^+-gradient in low gCl^- myotonia.

	Resting muscle	**Myotonic stiffness**	**Transient weakness**	**Warm-up**
K^+				
Osmolarity	290	290	330(e)	350(e)
BK (activation by large depolarization, high $[Ca^{+2}]_i$ and hyperosmolarity)	Ø	+	+	+
KCNQ5 (slow activation by depolarization)	Ø	Ø	+	+
NKCC1 (activation by hyperosmolarity)	Ø	Ø	Ø	+
Kir2.1 (activation by hyperpolarization)	Ø	Ø	Ø	(+)
Remark	Membrane instability due to low gCl^-	T-tubular K^+ accumulation, depolarization, AP bursts	Depolarization below -60 mV, AP-propagation block	Compensatory increase of gK^+ stabilizes membrane excitability

BK channels predominantly localize in the T-system and are activated by large depolarization, high $[Ca^{+2}]_i$ and hyperosmolarity (Siemer et al. 2000, Clerck et al. 2005, Kristensen et al. 2006). The results of this study show, that the BK-channel blocker paxilline prevented the onset of myotonic stiffness. The findings support data obtained from BK knock out mice (Sartorius et al. 2006). In terms of a potential clinical benefit of paxilline, it has to be considered that the drug also is an inhibitor of the SERCA type IIa leading to a decrease of Ca^{+2} reuptake into the SR (Tribe et al. 2000, Bilmen et al. 2002).

It may be speculated that an inhibition of K^+ efflux, which is causative for T-tubular K^+ accumulation and the electrical after-activity, prevents the onset of myotonia. The BK-agonist NS1608 led to a more severe relaxation deficit in experimental myotonia. However, a more rapid onset of warm-up was observed (see Fig. 25). This corroborates the view that K^+ clearance from the T-system might be enhanced via BK channels. Furthermore, the resulting hyperpolarization due to BK channel-activation may terminate the burst and thus reduce the hyperexcitability (Mallouk and Allard 2000).

KCNQ5 channels are activated with depolarization and enhance membrane repolarization (Schroeder et al. 2000). It has been reported that the potent agonist retigabine (100 µM) can yield a membrane hyperpolarization of up to 23.6 mV (Main et al. 2000). The interesting finding in the present study is that retigabine enhanced the warm-up phenomenon in human and murine samples. Hence, retigabine is an interesting candidate drug for potential clinical benefit in patients suffering from low gCl^- myotonia (Quasthoff et al. 1990, this study). This study failed to show that the KCNQ5 inhibitor XE991 shows the opposite effect of retigabine. A possible reason is that other channels compensate the lack of KCNQ5, or that the used concentration (30 µM XE991) was too low for our muscle preparations (Schwarz et al. 2006).

Warm-up occurs when $[K^+]$ does not further increase in the T-tubular lumen (i.e. AP-propagation block). However, due to the preceding strong activation of contractile filaments, there is an accumulation/efflux of acidic metabolites, which leads to an expansion of the T-tubular volume (Eisenberg and Gilai 1979, Usher-Smith et al. 2007). Osmolarity will first increase in the T-tubular compartment, then the metabolites and K^+ will slowly diffuse into the outer interstitial space and blood (Hess et al. 2005, Shorten and Soboleva 2007). This study shows, that increased osmolarity leads to a reduction of myotonic stiffness and to a sustained membrane depolarization. The myotonic burst rate was suppressed to a great extent. In fact, osmotic stress primary leads to activation of NKCC1. In the absence of gCl^- the inward transport of Cl^- via NKCC1 is essential. The

transporter causes membrane depolarization by enhancing Cl^--influx (Geukes Foppen et al. 2002, Ferenczi et al. 2003). In non-myotonic muscle NKCC1 accounts for more than 30% of K^+ import during muscle stimulation (Wong 2001, Zhao et al. 2004). It can be hypothesized that this fraction is higher in myotonic muscle because of overexpression of NKCC1. Data obtained with the NKCC1 inhibitor bumetanide corroborate this view. Bumetanide prevented the membrane depolarization and reversed the antimyotonic effect of high osmolarity. This effect was much more pronounced in muscle from ADR animals compared to the pharmacologically induced myotonic muscle. Hence, NKCC1 overexpression in low gCl^- myotonia is very likely and NKCC1 activation is one of the main mechanisms of the warm-up phenomenon. The loop diuretic bumetanide and derivatives, most notably furosemide, worsen the myotonic syndrome and must be avoided in patients with low gCl^- myotonia. The results of the NKCC1 experiments also support the clinical observation, that a nutritional increase of serum osmolarity (e.g. water deprivation, carbohydrate rich meals) has antimyotonic effects.

The Kir2.1 channel is the major skeletal muscle inwardly rectifying potassium channel, which is predominantly expressed in the T-system. Using a mathematic model, Wallinga et al. (1999) showed that the role of the Kir2.1 in maintaining excitability during repeated AP firing is almost as effective as the Na^+/K^+-ATPase. The Kir2.1 channels conduct inward current for T-tubular K^+ clearing if the potential is more negative than the K^+ equilibrium (Sejersted and Sjogaard 2000). Although the Kir2.1 channels conduct almost no outward current during depolarization, preventing large K^+ losses from active muscle, the channels also open when the equilibrium potential is a little more positive than E_K. Moreover, the channel takes some time to open and shut when the membrane potential changes (Aidley and Stanfield 1996). In myotonic T-tubules Kir2.1 might contribute to K^+-reuptake and restoration of the membrane excitability (Wallinga et al. 1999, Kristensen et al. 2006).

An effective Na^+/K^+-ATPase activity is essential for T-tubular function and accounts for 46% K^+ influx and quick restoration of $[K^+]_o$ to normal and even subnormal levels in non-myotonic muscle, thus hyperpolarizing the membrane by 3-10 mV (Drost et al. 2001, Nielsen et al. 2004, Zhao et al. 2004). Hence, it is tempting to speculate, that (over)activation of the Na^+/K^+-ATPase is an important mechanism of the warm-up phenomenon. However, neither agonists nor antagonists did influence myotonic stiffness or warm-up in low gCl^- myotonia (Nielsen et al. 2004, Colding-Jorgensen 2005, Van Beekvelt et al. 2006).

4.5 pH AND TEMPERATURE

Functional proteins like ion-channels strongly depend on pH and temperature. Acidic pH reduces gNa^+. Birnberger and Klepzig (1979) demonstrated that lowering external pH to 6.8 can abolish experimental myotonia in vitro, however, they did not directly measure intracellular pH. It is unlikely that pH drops below 7.0 in vivo, simply because of the large buffering capacity and intact perfusion. In murine muscle tetanic stimulation induced a shift of up to 0.2 units during extensive stimulation (Westerblad et al. 1992, Bruton et al. 1996, Broch-Lips et al. 2007). In fact, a decline of pH goes along with a marked increase of $[K^+]_o$ and vice versa (Pedersen et al. 2005). One speculation is, that there will be no direct effect of pH on the warm-up but rather an indirect effect via an increased K^+, which enhances the warm-up phenomenon. However, this question needs further investigation.

Intramuscular temperature and body core temperature have been discussed in the context of warm-up (Mano et al. 1985, McArdle et al. 1991). Some authors report variations of the myotonic features in low temperature (Bryant 1973, Fanning and MacDermott 1997). However, the data were generated in the premolecular era without genetic distinction of the entities. Up to date, there is no systematic in vitro study evaluating whether muscular hypothermia is a true trigger for low gCl^- myotonia. In patients suffering from low gCl^- myotonia, there is no increased stiffness after cooling (Ricker et al. 1977, Fournier et al. 2006).

4.6 FIBER COMPOSITION OF ADR MYOTONIC MUSCLE

Besides the electrical instability of the membranes, it has to be considered that the contraction performance is fiber type dependent. This study confirms that slow WT fibers (type I) need a lower frequency than the type II fast fiber to reach the full tetanus. In myotonic muscle the tetanus frequency was reduced by a factor of 1.3-2.1. These findings corroborate to histological data of ADR muscle.

In ADR myotonic mouse fibers, there is a switch from type IIb (glycolytic) into type IIa fibers. Type IIa fibers are oxidative and constitute 80% of total fibers in ADR muscle (Wu and Olson 2002). Also in the muscle biopsies of patients with low gCl^- myotonia, absence or a deficiency (less than 15%) of IIb fibers was observed (Crew et al. 1976, Heene et al. 1986). Based upon the metabolic profiles, type IIa fibers primarily rely on oxidative

phosphorylation to provide energy. This kind of fibers may be more resistant to strain than glycolytic fibers, which primarily use anaerobic glycolysis to generate ATP.

Muscle from myotonic humans and mice also have major changes in myosin heavy chains (MHCs) isoforms from MHC IIb to the slower MHC IIa that might worsen relaxation deficits and force build-up. The observation of lower tetanus frequencies in ADR mice supports this theory. One speculation is that a compensatory change to oxidative fibers occurs in myotonic muscle because of repetitive strenuous activation due to membrane hyperexcitability.

Moreover, it has been previously reported that the difference in the amounts/densities of K^+ regulating membrane proteins is also fiber type dependent. Kristensen et al. (2005) found that the relative amount of K^+ reuptake proteins (i.e. Kir2.1 channels and NKCC1) is lower in oxidative fibers compared with glycolytic fibers. This might explain why the most prominent loss of force (muscle weakness) during contraction was shown in oxidative fibers from ADR muscle.

4.7 MALIGNANT HYPERTHERMIA SUSCEPTIBILITY

Data obtained in the late 1980s suggested a link between low gCl^- myotonia and MH (Heiman-Patterson et al. 1988). However, these data were generated in the premolecular era without genetic distinction of the entities. Today, there is good evidence that low gCl^- myotonic muscle is not prone to true MH-events because functional testing for MH susceptibility is available (Newberg et al. 1983, Lehmann-Horn et al. 2007). The negative caffeine and halothane challenge in ADR-muscle of this study corroborate this view. This does not exclude the risk of an MH-like hypermetabolism during general anesthesia in low gCl^- myotonia. Depolarizing muscle relaxants might cause a severe myotonic episode with life-threatening hyperkalemia and rhabdomyolysis.

4.8 CLINICAL IMPLICATIONS

Patients can be quite successful in sports requiring more strength than speed because the muscle strength is greater than normal. Some affected members of Dr. Thomsen's family were able to make their living as trapeze acrobats (Colding-Jorgensen 2005, Lehmann-Horn et al. 2007). It is sometimes stated that gymnastics as such may have beneficial long-term effects.

The results of this dissertation (antimyotonic effects of high K^+ and high osmolarity) may have an impact on dietary counseling. However, K^+-levels in patients with low gCl^- myotonia should be adjusted to the upper physiological range. The study also helps to explain the beneficial effect of carbohydrate rich meals and the deleterious worsening of the myotonic symptoms during fasting. High blood glucose levels respectively high osmolarity promotes NKCC1 function. On the other hand NKCC1 inhibitors (furosemide, bumetanide) are inadvisable. If diuretics are necessary, K^+-saving substances are better for low gCl^- myotonia (e.g. spironolactone, amiloride, triamteren).

Whether nutritional Mg^{+2} supplementation has clinically relevant antimyotonic effects has not been investigated, however it seems to help to some degree (Mousain-Bosc et al. 2004, 2006, Kowalski et al. 2007). Muscle AP's can spread in retrograde direction along the peripheral motoneurons and thereby engage greater areas of muscle tissue. Mg^{+2} attenuates at least this nervous contribution to myotonic stiffness. If there is a specific effect on the sarcolemmal surface remains hypothetical (see above).

The $Na_v1.4$ channel blocker mexiletine is the drug of choice for patients suffering from myotonic syndromes (Lehmann-Horn et al. 2007). The drug inhibits the inward current (late Na^+ currents), and depresses the maximum rate of depolarization without affecting the resting potential, thus reducing the rate of AP (Wang et al. 2003). It has been shown that flecainide, another $Na_v1.4$ channel blocker, had similar effects (Desaphy et al. 2003, Wang et al. 2003). In this study both drugs dose-dependently decreased the relaxation deficit in ADR-muscle. Mexiletine exhibited a stronger effect at lower concentrations. This study confirms the antimyotonic effects of these drugs.

5 SUMMARY

Low chloride conductance (gCl^-) myotonia is a hereditary disease affecting skeletal muscle function. Mutations in the gene encoding the human skeletal chloride channel type 1 (CLCN1) cause electrical instability of the membranes and lead to a deficit in muscle relaxation. The phenotype shows muscle stiffness followed by transient weakness. By unknown mechanisms, this type of myotonia shows a reduction of stiffness after exercise, the so-called warm-up phenomenon. This study investigated myotonic stiffness and the warm-up phenomenon in vitro. Mechanographic registrations and membrane potential measurements were performed. Muscle samples from the animal model for low gCl^- myotonia (ADR-mice) as well as pharmacologically induced myotonia were studied. The microenvironmental conditions, i.e. $[K^+]_o$ and osmolarity were varied and pharmacological substances with potential clinical benefit were tested.

In low gCl^- myotonia, the resting membrane potential (RMP) is set solely by the K^+ conductance (gK^+). At physiological conditions (3.5 mM $[K^+]_o$, 290 mOsm), the RMPs were -79.6 $\pm$ 2.3 mV for control and -75.8 $\pm$ 2.6 mV for ADR.

Strong contraction triggers K^+ accumulation in the small T-tubular compartment shifting the membrane potential towards the activation threshold of voltage gated Na^+ channels ($Na_v1.4$). This effect is capable to initiate self-sustaining bursts of action potentials (i.e. myotonic bursts), thereby prolonging muscle relaxation (myotonic stiffness). The relaxation deficit was shown in single twitch and tetanic contraction in this study.

Further increase in K^+ led to a sustained membrane depolarization in a concentration-dependent manner. At 7 mM $[K^+]_o$ the membrane potential was shifted to -60.3 $\pm$ 3.2 mV in control, and -59.3 $\pm$ 2.8 mV in ADR-mice and the myotonic burst rate was suppressed more than 90%. Under hyperosmotic conditions (e.g. 400 mOsm) there also was a shift towards less negative values by 15.7 $\pm$ 3.6 mV for control fibers and by 6.5 $\pm$ 2.3 mV for ADR samples. Again the myotonic burst rate was suppressed to a great extent (50%). The large depolarization forces enough $Na_v1.4$ channels into inactivation. Thereafter, the action potential (AP) propagation into the T-system is blocked and the T-tubular membrane is temporarily hypoexcitable. This is the basis of transient weakness, which was observed in ADR isolated muscle within 1-3 s after initiation of full tetanus-frequency stimulation.

Repetitive contractions alleviated the relaxation deficit as well as the transient weakness. The warm-up phenomenon reflects an enhanced stabilization of the membrane excitability. Potential mechanisms are activation of proteins compensating the lack of gCl^- in order to

reduce T-tubular K^+ accumulation and/or increase the membrane leak conductance. The T-tubular membrane is characterized by a high expression level of big conductance calcium activated potassium channels (BK). BK is activated by an increase of intracellular [Ca^{2+}] and depolarization. Hence, K^+-efflux via BK-channels contributes to the T-tubular K^+ accumulation until a new electrochemical equilibrium is reached in the T-system. This study shows that the BK-agonist NS1608 led to a more severe relaxation deficit, but also to a more rapid onset of warm-up. Blocking the BK channel with paxilline prevented the onset of myotonia, probably due to a reduction of T-tubular K^+ accumulation.

Another interesting finding in this dissertation is the fact, that increasing gK^+ via activation of the voltage gated potassium channels (KCNQ5) by retigabine enhanced the warm-up phenomenon. Taking together the results, an increased gK^+ accelerates repolarization and stabilizes the RMP.

Upon osmotic stress ADR muscle was depolarized to a lesser extent than control; that might suggest in a compensatory alteration of the protein expression pattern in ADR muscle. Osmotic stress primarily leads to activation of the sodium potassium chloride cotransporter type 1 (NKCC1). The NKCC1 inhibitor bumetanide prevented the depolarization and reversed the antimyotonic effect of high osmolarity. Hence, T-tubular ion-homeostasis is restored by enhancing Cl^--inward transport through the NKCC1. Therefore, the NKCC1 is one of the main mechanisms in the warm-up phenomenon.

Blocking $Na_v1.4$ channels with mexiletine and flecainide dose-dependently facilitated the warm-up phenomenon in ADR muscle. These results confirm in vivo observations of antimyotonic effects of $Na_v1.4$ inhibitors.

The findings have an important impact on dietary counseling (e.g. K^+ rich meals, K-saving diuretics) and the use of drugs influencing gK^+ and gNa^+. These may have clinical relevance towards improved management and treatment of myotonic stiffness in low gCl^- myotonia.

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7 LISTS OF TABLES AND FIGURES

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