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Restricted diffusion of ions regulates cardiac function

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... at last!

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I could not have finalized the papers in this thesis without considerable help from my co-authors: Bjørn Amundsen, Jan Magnus Aronsen, Jon Arne Birkeland,

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1. ABBREVIATIONS

[ion or substrate] – concentration of ion or substrate

[ion]$_i$ – intracellular ion concentration

[ion]$_o$ – extracellular ion concentration

[ion]$_{ss}$ – ion concentration in submembrane space

ADP – adenosine diphosphate

AM - acetoxymethyl

ATP – adenosine triphosphate

CaMKII - Ca^{2+}-calmodulin dependent protein kinase II

CHF – congestive heart failure

CICR – Ca^{2+} induced Ca^{2+} release

cSEVC - continuous single electrode voltage clamp

DAD – delayed afterdepolarizations

DHO – dihydro-ouabain

dSEVC - discontinuous single electrode voltage clamp

$I_{Ca,L}$ – L-type calcium current

I_{K1} – inward rectifier potassium current

I_{Kr} – delayed rectifier potassium current

I_{Na} – sodium current

I_{NCX} – Na^+/Ca^{2+}-exchanger current

I_{NKA} – Na^+/K^+-ATPase current

I_{to} - transient outward potassium current

$K_{0.5}$ – half-maximal activation constant (affinity)

LVEDP - left ventricular end diastolic pressure

NCX – Na^+/Ca^{2+}-exchanger

NKA – Na^+/K^+-ATPase

P_i – free phosphate

PKA – protein kinase A

PKC – protein kinase C

RyR – ryanodine receptor

SBFI - sodium-binding benzofurzan isophthalate

SERCA – sarcoendoplasmic reticulum calcium ATPase

SR – sarcoplasmic reticulum

V_{max} – maximal pump rate

2. LIST OF PAPERS

Paper 1:

Slow diffusion of K^+ in the t-tubules of rat cardiomyocytes

Journal of Applied Physiology 2006;101:1170-6.

Fredrik Swift, Tævje A. Strømme, Bjørn Amundsen, Ole M. Sejersted, Ivar Sjaastad

Paper 2:

The Na^+/K^+-ATPase α_2-isoform regulates cardiac contractility in rat cardiomyocytes

Cardiovascular Research 2007;75:109-17.

Fredrik Swift, Nils Tovsrud, Ulla H. Enger, Ivar Sjaastad, Ole M. Sejersted

Paper 3:

Altered regulation of Na^+/Ca^{2+}-exchanger activity due to downregulation of Na^+/K^+-ATPase α_2-isoform in cardiomyocytes from rats with post infarction congestive heart failure

Submitted manuscript

Fredrik Swift, Jon Arne Kro Birkeland, Nils Tovsrud, Ulla H. Enger, Jan Magnus Aronsen, William E. Louch, Ivar Sjaastad, Ole M. Sejersted

3. INTRODUCTION

3.1. Cardiac function

3.1.1. The normal heart

The heart is responsible for pumping blood in the circulatory system allowing transport of nutrients, water, oxygen, carbon dioxide, etc. in the body. The heart must constantly regulate its contractile force to meet the variable demands of the body. In skeletal muscles, contractile force is adjusted by recruiting more or fewer muscle fibres. However, in the heart this is not possible since all the cardiomyocytes are electrically coupled. Therefore, contractility must be adjusted at the level of the individual cardiomyocyte.

Developed force in single cardiomyocytes is regulated by a multitude of mechanisms. The concerted action of these mechanisms constitutes what is known as the excitation-contraction-relaxation coupling and converts the electrical stimulus of the cardiomyocyte to mechanical response. A key process in the excitation-contraction-relaxation coupling is transport of ions across the sarcolemma through ion transporting proteins and within the cardiomyocyte. In recent years, several
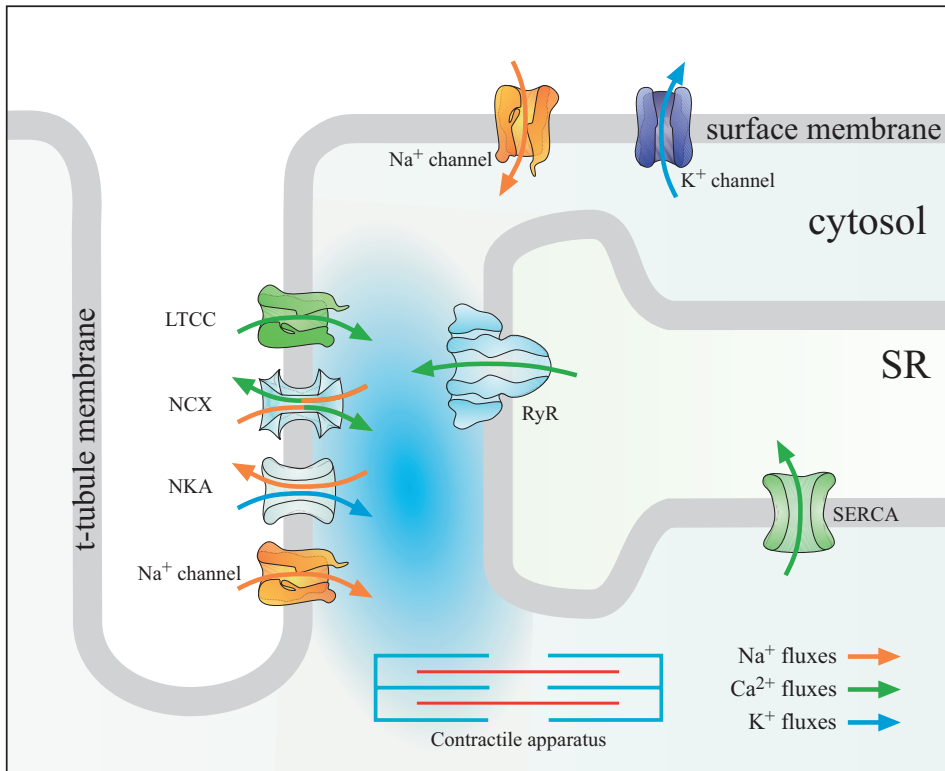
Figure 1. *Schematic representation of key proteins in the excitation-contraction-relaxation coupling.* These proteins cooperate to transport ions during the cardiac cycle. LTCC, L-type Ca^{2+} channel; NCX, Na^+/Ca^{2+}-exchanger; NKA, Na^+/K^+-ATPase; RyR, Ryanodine receptor, SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum.

observations have lead researchers to propose that diffusion of ions in cardiomyocytes is slower than in water. It follows that ion concentrations can be different in various spaces in the cardiomyocyte. For example, the concentration of Na^+ would be much higher at the mouth of an open Na^+ channel than in the bulk cytosol [149]. With diffusion of ions restricted to discrete spaces, excitation-contraction-relaxation coupling seems to occur without the need of individual ions to travel very far. This is reflected in the morphology of cardiomyocytes which have invaginations of cell membrane called t-tubules. T-tubules connect the excitable membrane to the

sarcoplasmic reticulum (SR), which is an intracellular Ca^{2+} store. An influx of Ca^{2+} over the t-tubule membrane can thus locally trigger a larger release of Ca^{2+} from the SR which will trigger the contraction. Figure 1 shows an overview of key proteins participating in the excitation-contraction-relaxation coupling and their respective ion fluxes. Recent advances in excitation-contraction-relaxation coupling research have demonstrated that Na^+ plays an important role as a regulator for Ca^{2+} handling. However, the mechanisms behind this regulation are still unclear. The excitation-contraction-relaxation coupling will be presented in more detail below.

3.1.2. The failing heart

Heart failure is a common disease, with more than 550 000 new cases per year in the US alone. It is primarily a condition in the elderly with ~80% of patients being above the age of 65. Thus, aging of the population contributes to increased prevalence and an increasing number of hospitalizations. Despite advances in the treatment of the disease, the number of deaths from heart failure has increased steadily during recent years. One probable cause for this, is that better treatment and early interventions rescues more patients from dying from myocardial infarctions earlier in life [62]. However, these patients later develop heart failure.

Heart failure may have various etiologies including hypertension, myocardial infarction, valvular heart disease and cardiomyopathies, and has been defined as "a pathophysiological state in which the heart is unable to pump blood at a rate commensurate with the requirements of the metabolizing tissues or can do so only from an elevated filling pressure" [26]. It is often described as a *systolic* or *diastolic* heart failure. During systolic heart failure, the ability of the heart to eject blood during systole is impaired. In contrast, systolic function can be preserved during diastolic heart failure in which diastolic filling of the ventricle is impaired. Ventricular stiffening

is an important contributor to diastolic heart failure which is particularly prevalent in older patients, women and in patients with hypertension [27]. Heart failure caused by chronic ischemic heart disease is the most common form and is often a combined systolic and diastolic heart failure [27].

In the acutely failing heart following a large infarct, function of surviving myocardium might be normal, or even enhanced. However, the myocardium is gradually remodelled after an index event. Remodelling comprises both morphological and functional aspects, and generally results in reduced cardiac function. First, structural remodelling can develop at the macroscopic level with left ventricular dilatation and/or myocardial fibrosis and reorganization. Second, structural remodelling of the cardiomyocytes develops during chronic congestive heart failure (CHF), as discussed in the present thesis. Third, myocardial function is altered, although both enhanced [88] and reduced [122;147] function has been observed. Also, control of intracellular Na^+ concentration is reduced in the remodelled myocardium (discussed later in this thesis). Remodelling of the left ventricle results in reduced cardiac performance in the chronic failing heart.

The contractile defect observed in myocardial heart failure in rats can be quantified *in vivo* by reduced fractional shortening and reduced shortening velocity [123;124]. This defect is also commonly observed in isolated cardiomyocytes [121], but not in all experiments [88]. Also delayed relaxation is a common finding in failing cardiomyocytes [124;147]. These defects can in part be explained by alterations in excitation-contraction-relaxation coupling [20].

3.2. Excitation-contraction-relaxation coupling

3.2.1. The action potential

Excitation-contraction-relaxation coupling is initiated by an action potential. The action potential is a transient change in the membrane potential, resulting from an intricate interplay between several ion transporting proteins. It propagates in the cardiomyocyte by a combination of facilitated diffusion of Na^+ through open voltage-gated Na^+ channels and passively spreading membrane depolarization. When Na^+ channels open in a region of cell membrane, positive charges (Na^+) enters the cell. By electrostatic repulsion, adjacent ions are "pushed" through the cytoplasm, resulting in a wave of positivity to downstream regions. This depolarizes the neighbouring membrane to the threshold value for opening of Na^+ channels, and the process repeats itself [12]. The propagation from cell to cell is permitted by gap junctions in the intercalated discs of the cardiomyocytes. It is important to point out that by this propagation mechanism, individual ions do not need to be moved very far.

The action potential can be described as four successive phases:
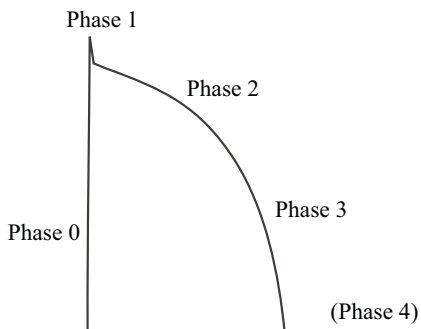
Figure 2. *The phases of the action potential.*

Phase 0 is a depolarizing phase where Na^+ flows into the cell through Na^+ channels. During this phase, the membrane potential changes from its resting value of ~-80 mV and reaches typically +35-50 mV [12]. The Na^+ channels inactivates within ~1 ms, so despite a very large peak Na^+ current (I_{Na} >1 nA/pA [12]), the amount that enters can only raise bulk intracellular Na^+ concentration ($[Na^+]_i$) by 6-15 µM [15]. Yet, because of slow diffusion of Na^+ in the cytosol, local $[Na^+]$ in the subsarcolemmal space ($[Na^+]_{ss}$) during I_{Na} may be 60 times higher than bulk $[Na^+]_i$ [149].

Phase 1 is an early repolarizing phase mainly carried by a transient outward K^+ current (I_{to}). Differences in the repolarizing phase are seen between species, but also between cardiomyocytes from different regions of the ventricle, and have been explained by varying amounts of I_{to} [141]. Because the early repolarization phase is so great in rat and mouse ventricular cardiomyocytes, they have almost no plateau phase (see below). During this phase, the action potential is more positive than the reversal potential for a Na^+/Ca^{2+}-exchanger which then operates in the its reverse mode, bringing Ca^{2+} into the cell [69].

Phase 2 is a plateau phase where the membrane potential changes slowly. During this phase, inward currents mainly through L-type Ca^{2+} channels are almost in balance with outward currents carried by delayed rectifier K^+ channels (I_{Kr}). The Ca^{2+} coming in over the sarcolemma will trigger a larger release of Ca^{2+} from the SR by binding to SR Ca^{2+} release channels, or ryanodine receptors (RyR), by a process called Ca^{2+} induced Ca^{2+} release (CICR) [46]. This Ca^{2+} binds to troponin C in the myofilaments, causing the contraction of the cardiomyocyte.

Phase 3 is a repolarizing phase. This phase is mainly driven by K^+ channels. As repolarization proceeds, outward current through inward rectifier K^+ channels (I_{K1}) and delayed rectifier K^+ channels (I_{Kr}) increases, and brings the potential towards the

resting potential. During this phase, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is still relatively high. Together with the low membrane potential, this favours forward mode Na^+/Ca^{2+}-exchange (Ca^{2+} extrusion).

A fifth phase (phase 4) is also sometimes described. In ventricular cardiomyocytes, this phase consists of the resting period between action potentials, where the membrane potential is held at ~-80 mV. This phase is maintained mainly by I_{K1} until a new action potential is triggered. In cells with spontaneous pacemaker activity, there is a gradual depolarization during this phase. These cells have little or no I_{K1} [12]. When the membrane potential reaches threshold values for I_{Na}, a new action potential is elicited.

During heart failure, the action potential is prolonged. This has been attributed to a reduction in I_{to} [137]. Since I_{to} profoundly influences phase 1 and the level of the plateau, it also affects all the other currents that are active later in the action potential. One protein of which the function is highly dependent on the membrane potential is the Na^+/Ca^{2+}-exchanger. Prolongation of the plateau phase of the action potential could prolong the period for Ca^{2+} influx [44], or at least reduce the Ca^{2+} efflux via Na^+/Ca^{2+}-exchangers. This would contribute to the delayed relaxation seen in heart failure.

3.2.2. Triggers of Ca^{2+} release

Already in 1883, Sydney Ringer discovered that cardiac muscle contraction is dependent on extracellular Ca^{2+} [101]. Indeed, Ca^{2+} must enter the cardiomyocyte to trigger a larger release from the SR. This occurs by two mechanisms: through L-type Ca^{2+} channels and to a lesser extent by reverse mode Na^+/Ca^{2+}-exchange.

The L-type Ca^{2+} channel is a voltage dependent channel which passes Ca^{2+} current ($I_{Ca,L}$) when it is open. The $I_{Ca,L}$ constitutes the main trigger mechanism for SR

Ca^{2+} release and is dependent on membrane potential in a bell-shaped manner. Maximum $I_{Ca,L}$ is reached at ~0 mV, with little or no current at potentials negative to -40 mV and positive to +40 mV. During Phase 1 of the action potential, L-type Ca^{2+} channels open rapidly and $I_{Ca,L}$ reaches a peak within 2-7 ms [12]. The $I_{Ca,L}$ is sustained through phase 1 and 2, but then declines. The inactivation of the L-type Ca^{2+} channel is dependent on time, membrane potential and $[Ca^{2+}]_i$. Thus, Ca^{2+} constitutes a negative feedback signal to close the L-type Ca^{2+} channel. The amount of Ca^{2+} that enters the cardiomyocyte is dependent on both activation and inactivation of $I_{Ca,L}$. Since the action potential is shorter in the rat than in other species, the influx of Ca^{2+} through $I_{Ca,L}$ during each cycle is also smaller in the rat; 14 μmol/l cytosol (vs. 21 μmol/l cytosol in rabbits) [12]. However, important Ca^{2+} buffers are present in the cytosol, so an entry of 14 μmol/l cytosol would result in a much smaller rise in free $[Ca^{2+}]_i$ (ratio of 90:1 [9;12]). On the other side, it now seems clear that local subsarcolemmal Ca^{2+} ($[Ca^{2+}]_{ss}$) in the dyadic cleft can be raised from 0.1 μM to more than 10 μM by $I_{Ca,L}$ [18]. For this to occur, diffusion of Ca^{2+} must be restricted to a submembrane space. The increase in Ca^{2+} triggers a larger release of Ca^{2+} from the SR (see below).

In most models of heart failure, peak $I_{Ca,L}$ density is found to be unaltered [122;124]. However, the ability of $I_{Ca,L}$ to trigger contractions is still a matter of debate [54;122]. At the single channel level, it has been reported that the opening probability and availability of L-type Ca^{2+} channels are increased in human heart failure [107]. However, counterbalanced by a reduced number of L-type Ca^{2+} channel protein copies in heart failure [59], this fits with unaltered peak $I_{Ca,L}$ density, but could result in reduced trigger efficiency. In line with this, a defective interaction between the L-type Ca^{2+} channel and RyR has been proposed in heart failure [53]. It has also

been proposed that remodelling of t-tubules leads to orphaned RyR [128]. Thus, it appears to be a marked decrease in the ability of the L-type Ca^{2+} channel to activate RyRs in heart failure [32]. In other words, the gain of CICR may be reduced if the L-type Ca^{2+} channel protein expression or cardiomyocyte micro-architecture is altered.

Reverse mode Na^+/Ca^{2+}-exchange, bringing Ca^{2+} into the cardiomyocyte, was proposed as a trigger for SR Ca^{2+} release for the first time in 1990 [69]. Since then, several studies have confirmed this role of the Na^+/Ca^{2+}-exchanger [74;102]. The major part of Na^+/Ca^{2+}-exchangers is located in the t-tubules [39]. It has been shown that a fraction of the Na^+/Ca^{2+}-exchangers are localized as close to the RyR as the L-type Ca^{2+} channel [136] (but this is also disputed [109]). This is compatible with a triggering role for reverse Na^+/Ca^{2+}-exchanger activity. The reversal of the Na^+/Ca^{2+}-exchanger requires a combination of positive membrane potential and increased $[Na^+]_{ss}$. The reversal potential for the Na^+/Ca^{2+}-exchanger is typically -30 to -80 mV at rest [12]. Thus, during the action potential, the Na^+/Ca^{2+}-exchanger will reverse and cause Ca^{2+} influx. Several reports show that this Ca^{2+} entry can trigger SR Ca^{2+} release and contractions [5;148], even in the absence of $I_{Ca,L}$ [72]. During the initial phase of the action potential, Na^+ will flow into the cell through Na^+ channels. Thus, Na^+ can accumulate in subsarcolemmal spaces adjacent to Na^+/Ca^{2+}-exchangers, facilitating Ca^{2+} influx [70;77]. In a recent study it was shown that reverse mode Na^+/Ca^{2+}-exchange participates in early trigging of SR Ca^{2+} release, before trigging by $I_{Ca,L}$. It was predicted by a mathematical model that this was only possible if a Na^+ channel was present in the dyad and if diffusion of Na^+ in the dyad was slow [74]. In a recent report it was concluded that I_{Na} might slightly enhance Ca^{2+} influx through Na^+/Ca^{2+}-exchangers in the early phase of the action potential, consistent with a role for Na^+/Ca^{2+}-exchanger in early trigging [149]. Further, other mechanisms regulating

$[Na^+]_{ss}$, such as the Na^+/K^+-ATPase, might be a determinant for the role of reverse

mode Na^+/Ca^{2+}-exchange. Supporting this, it has been shown that the Na^+/K^+-

ATPase modulates the influence of I_{Na} on Ca^{2+} transients [129].

Despite all the current data, the physiological role of reverse mode Na^+/Ca^{2+}-

exchange as a trigger of SR Ca^{2+} release is still discussable. What seems clear,

however, is that it might play an important role in heart failure. Indeed, several

models of heart failure are associated with prolonged action potentials, increased

$[Na^+]_i$ and smaller Ca^{2+} transients, all of which will favour reverse mode Na^+/Ca^{2+}-

exchange activity [13]. Also, increased expression of the Na^+/Ca^{2+}-exchanger in heart

failure could influence trigging of SR Ca^{2+} release.

3.2.3. Intracellular Ca^{2+} handling

Trigger Ca^{2+} binds to RyRs, which then open and release a substantially larger

amount of Ca^{2+} from the SR. The ensuing transient rise in $[Ca^{2+}]_i$ allows binding of

Ca^{2+} to troponin C in the myofilaments which causes actin and myosin filaments to

interact and produce force. For relaxation to occur, the Ca^{2+} that came in over the

sarcolemma as a trigger must be pumped back out of the cell, and Ca^{2+} released

from the SR must be pumped back into the SR. These processes are summarized in

Figure 3.
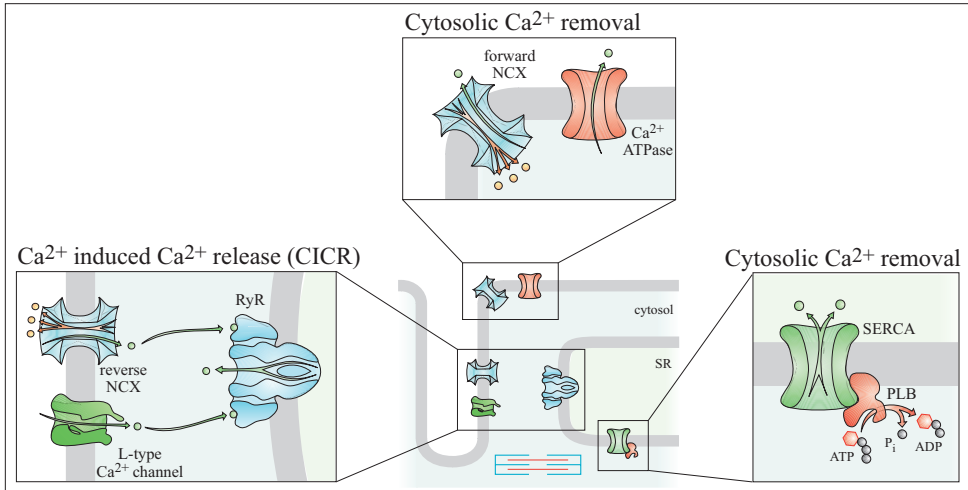
Figure 3. *Ca^{2+} handling in cardiomyocytes*. A section of the cardiomyocyte including the surface sarcolemma, a t-tubule and the sarcoplasmic reticulum (SR) is shown. The left panel shows how Ca^{2+} enters through L-type Ca^{2+} channels and reverse mode Na$^+$/Ca^{2+}-exchangers, binds to ryanodine receptors (RyRs) and triggers a release of Ca^{2+} from the SR. The top panel shows how Ca^{2+} is extruded from the cell through forward mode Na$^+$/Ca^{2+}-exchanger and through the sarcolemmal Ca^{2+} ATPase. The right panel shows how Ca^{2+} is pumped back into the SR by SERCA.

Figure 4. *The Ca^{2+} transient.*

Top panel: averaged Ca^{2+} transient from a confocal line scan image.

Bottom panel: confocal line scan image from a field stimulated cardiomyocyte. Individual Ca^{2+} sparks can be observed as bright spots during diastole (arrows).
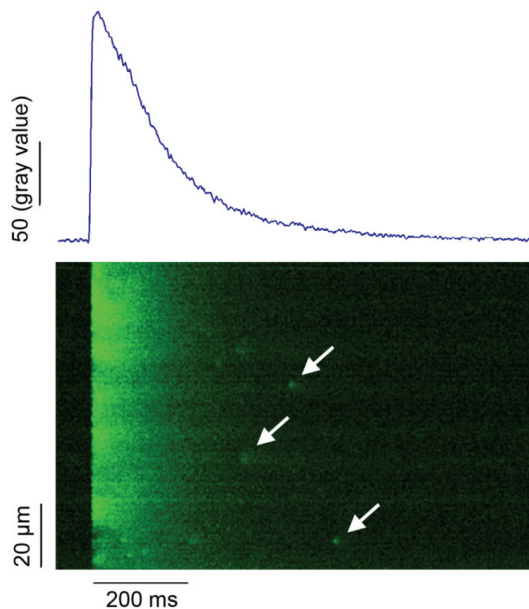
SR Ca^{2+} release

L-type Ca^{2+} channels are present in the whole sarcolemma, but are more abundant in the t-tubules in proximity to RyRs located in the SR membrane [33;49]. Indeed, the t-tubule membrane is apposed to the SR membrane in highly specialized junctional microdomains. Here, L-type Ca^{2+} channels face RyRs with a stoichiometry of 4-10 RyR for each L-type Ca^{2+} channel, depending on species [14]. The two adjacent membranes are separated by a cleft of 10-15 nm which is called the dyadic cleft. Clusters of individual RyRs and L-type Ca^{2+} channels separated by a dyadic cleft constitutes a functional unit called a couplon, or dyad [18]. The number of RyRs in one couplon is still a matter of debate; numbers from 6 to 40 are likely, although as many as 200 has been reported [12;64]. When one or several L-type Ca^{2+} channels open [64], Ca^{2+} release is triggered from the RyRs in that couplon by an amplification system (CICR). This can be observed as a Ca^{2+} spark in experiments [36;90] (see Figure 4). However, Ca^{2+} from one dyad does not normally diffuse to the next dyad. When an action potential travels through the cardiomyocyte, several individual Ca^{2+} sparks are triggered simultaneously and their spatiotemporal summation constitute the Ca^{2+} transient. Thus, it is the number of sparks recruited during an action potential (i.e. the number of open L-type Ca^{2+} channels) that determines the amplitude of the whole-cell Ca^{2+} transient. However, the degree of amplification in CICR depends on the amount of Ca^{2+} stored in the SR: the SR Ca^{2+} load [115].

SR Ca^{2+} load

The SR Ca^{2+} load at any given time point is determined by the balance between pumping of Ca^{2+} into the SR through a SR Ca^{2+} ATPase, SERCA, and Ca^{2+} efflux from the SR through RyRs. At steady-state, the amount of Ca^{2+} released through

RyR during a transient equals the amount pumped back by SERCA. Likewise, trigger Ca^{2+} that flows into the cell over the sarcolemma is extruded through forward mode Na^+/Ca^{2+}-exchange (with a small contribution from a Ca^{2+} ATPase in the sarcolemma). Forward mode (Ca^{2+} extrusion) is the main exchange mode of the Na^+/Ca^{2+}-exchanger. It follows that SERCA and Na^+/Ca^{2+}-exchangers compete to remove cytosolic Ca^{2+}. Therefore, when Na^+/Ca^{2+}-exchanger activity is altered, this will influence the SR Ca^{2+} load. At increased influx of Ca^{2+} over the sarcolemma, SERCA will pump more Ca^{2+} into the SR, leading to an increase in the SR Ca^{2+} load. This occurs within only one or two beats [115]. The increased SR Ca^{2+} load sensitizes the RyRs towards Ca^{2+} triggers from the cytosolic side [55]. The result is increased amplitude of the Ca^{2+} transient which will in turn lead to increased forward mode Na^+/Ca^{2+}-exchange and reduced Ca^{2+} influx through L-type Ca^{2+} channels due to increased Ca^{2+} dependent inactivation. Together, these mechanisms will normalize SR Ca^{2+} load [139]. Thus, the SR Ca^{2+} load is continuously regulated by a negative feedback mechanism where the Na^+/Ca^{2+}-exchanger is an important determinant [142]. However, the most important regulator of SR Ca^{2+} load is SERCA, which will be discussed below.

It is well established that the transport rate and exchange mode of the Na^+/Ca^{2+}-exchanger is controlled by intracellular concentrations of Na^+ and Ca^{2+} as well as the membrane potential. Therefore, control of intracellular Na^+ is essential for the cardiomyocyte in order to maintain normal contractility. A main regulator of $[Na^+]_i$ is the Na^+/K^+-ATPase. Therefore, the Na^+/K^+-ATPase can influence SR Ca^{2+} load. When $[Na^+]_i$ increases, this will change the driving force of the Na^+/Ca^{2+}-exchanger, reducing Ca^{2+} extrusion through forward mode exchange. Blockade of the Na^+/K^+-ATPase by cardiac glycosides (digitalis, digoxin) will therefore increase the

SR Ca^{2+} load, and therefore also Ca^{2+} transients and contractions [12]. However, at toxic levels cardiac glycosides will result in excessive amounts of Ca^{2+} in the SR (Ca^{2+} overload). Ca^{2+} overload of the SR will increase the opening probability of RyR [36], leading to an increased leak of Ca^{2+} through RyRs [116]. This can cause spontaneous Ca^{2+} release and arrhythmias. Spontaneous openings of RyR in diastole, which also have been shown to occur during heart failure, will release Ca^{2+} which will be extruded through the Na^+/Ca^{2+}-exchanger. This extrusion causes a transient inward current (since the Na^+/Ca^{2+}-exchanger is electrogenic, transporting 3 Na^+ for 1 Ca^{2+}), responsible for depolarizing the membrane towards the threshold for triggering of action potentials [98]. This can cause delayed afterdepolarizations (DADs) [16]. It follows that the Na^+/Ca^{2+}-exchanger has a dual role in the generation of DADs: 1, it controls the SR Ca^{2+} load, and 2, it carries the depolarizing current [142]. Thus, correct balance of the Na^+/Ca^{2+}-exchanger is important for maintaining cardiac function, particularly during heart failure when spontaneous openings of the RyR are likely to occur due to hyperphosphorylation [83] (see below).

SR Ca^{2+} reuptake

The amount of Ca^{2+} released from the SR is larger than the amount of Ca^{2+} entering over the sarcolemma. Therefore, at steady state, more Ca^{2+} has to be pumped back into the SR than out of the cell. Thus, despite the important role for the Na^+/Ca^{2+}-exchanger in the regulation of SR load, the contribution of SERCA in removing Ca^{2+} from the cytosol is considerably greater than that of the Na^+/Ca^{2+}-exchanger. The relative contribution of SERCA vs. Na^+/Ca^{2+}-exchanger and sarcolemmal Ca^{2+} ATPase varies between species, and has been estimated to 70% in rabbits, and to 92% in rodents [8]. It follows that SERCA is the main determinant for the kinetics of

individual Ca^{2+} transients [142]. SERCA is the centre of a macromolecular complex also containing regulatory proteins such as phospholamban, cAMP-dependent protein kinase A (PKA), Ca^{2+}-calmodulin dependent protein kinase II (CaMKII) and protein phosphatases PP1 and PP2A. Its activity is determined by the $[Ca^{2+}]_i$ and the SR Ca^{2+} load and is regulated by phospholamban [43]. An increase in $[Ca^{2+}]_i$ will increase the activity of SERCA whereas increased SR Ca^{2+} load means that SERCA must pump against a steeper Ca^{2+} gradient, slowing the pumping rate [63]. The Ca^{2+} affinity of SERCA is regulated by phospholamban, which can be phosphorylated by kinases and dephosphorylated by phosphatases. When dephosphorylated by PP1 or PP2A, phospholamban inhibits SERCA. Upon phosphorylation by PKA at Ser16 or CaMKII at Thr17, a conformational change in the protein structure relieves this inhibition. The consequence of a reduced SERCA activity is reduced SR Ca^{2+} load, resulting in a smaller Ca^{2+} transient. Due to this regulation by phosphorylation, SERCA activity is modulated by the β-adrenergic system. A full β-adrenergic effect can be mediated by PKA phosphorylation at Ser16. Phosphorylation at Thr17 by CaMKII may only occur after sufficient rise in $[Ca^{2+}]_i$ and is additive to the effect of PKA phosphorylation [140].

Defects in intracellular Ca^{2+} handling during heart failure

In heart failure, most studies report decreased SR Ca^{2+} load [60;73;96;97]. There are at least three possible explanations for this: (1) Reduced SERCA function. This could be due to reduced amounts of SERCA protein, but such reduction is controversial [48]. More likely, reduced phosphorylation of phospholamban underlies decreased SERCA activity in heart failure [48;104]. Reduced phosphorylation of phospholamban can partially be explained by the downregulation of β-adrenergic receptors in heart failure, but also by increased activity of PP1 and PP2A [140]. (2) Increased forward

mode Na^+/Ca^{2+}-exchange. Several studies report 50-100% increased levels of Na^+/Ca^{2+}-exchanger mRNA, protein and current (I_{NCX}) [12]. Higher forward mode Na^+/Ca^{2+}-exchange will compete better with SERCA during relaxation. This is supported by studies of ventricular cardiomyocytes with overexpressed levels of Na^+/Ca^{2+}-exchanger showing depressed contractility and reduced SR Ca^{2+} load [106]

(3) Increased leak of Ca^{2+} from the RyR. It has been reported that RyR can be hyperphosphorylated by PKA in heart failure, increasing the open probability of RyR and thus reducing SR Ca^{2+} load [82;83]. However, these results are controversial [17;152] and were recently contested in a study showing that increased Ca^{2+} leak through RyR was mediated through β-adrenergic stimulation of CaMKII, independently of PKA or bulk $[Ca^{2+}]_i$ [38]. In heart failure, both the amount and activity of CaMKII are upregulated, resulting in enhanced RyR phosphorylation and diastolic SR Ca^{2+} leak [2]. The physiological importance of leaky RyRs is still unclear. The effect of increased RyR leak has been challenged by the demonstration that modulation of the systolic function of RyR only causes transient changes in SR Ca^{2+} release [45]. It was recently also shown that increased RyR open probability could not produce SR Ca^{2+} leak unless SR Ca^{2+} content was increased [143]. Thus, since leak actually reduces the SR Ca^{2+} load, leaky RyRs could help prevent SR Ca^{2+} overload [120]. Nevertheless, leaky RyRs could participate in the genesis of triggered arrhythmias (DADs) as described above, especially if SR Ca^{2+} load is increased [143].

3.2.4. Role of intracellular Na^+

The $[Na^+]_i$ in cardiomyocytes is tightly regulated and results from the balance between Na^+ influx mechanisms and Na^+ efflux mechanisms. Na^+ influx occurs through numerous mechanisms, primarily through Na^+ channels and the Na^+/Ca^{2+}-exchanger, but also through the Na^+/H^+-exchanger, co-transporters of Na^+/HCO_3^-,

$Na^+/K^+/Cl^-$ and Na/Cl^-, and Na^+/glucose and Na^+/amino acid carriers [71]. The main mechanism for Na^+ efflux is the Na^+/K^+-ATPase, which is essential for keeping $[Na^+]_i$ low. The activity of the Na^+/K^+-ATPase can be adjusted to face an altered influx of Na^+ over the sarcolemma. This occurs primarily by changing the affinity of the Na^+/K^+-ATPase for $[Na^+]_i$, but also by changing the maximal pump rate (V_{max}) of the Na^+/K^+-ATPase. The principles for regulation of $[Na^+]_i$ are presented in Figure 5 where the transport rate of the Na^+/K^+-ATPase is plotted as a function of $[Na^+]_i$ (dark blue line). At increasing Na^+ influx (red arrow), the Na^+/K^+-ATPase will adjust its activity at a new higher $[Na^+]_i$. Increased intracellular Na^+ affinity ($K_{0.5}$) of the Na^+/K^+-ATPase is represented by the leftward shifted dashed blue line. A study showed that in Na^+ loaded cardiac Purkinje fibres, both V_{max} and $[Na^+]_i$ affinity were increased after addition of Ca^{2+} [114]. This increased the Na^+ efflux through the Na^+/K^+-ATPase. These results show that recovery from high intracellular Ca^{2+} is closely linked to reduction of $[Na^+]_i$.
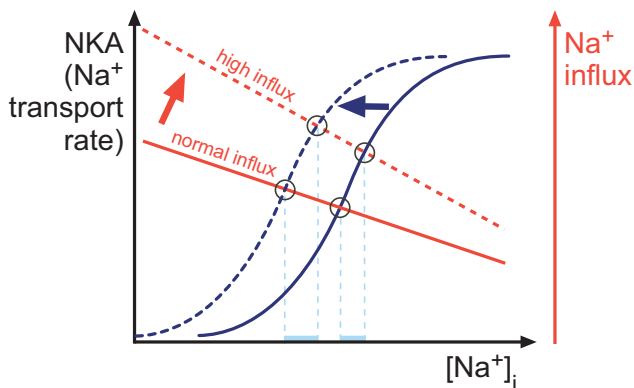
Figure 5. *Principles of regulation $[Na^+]_i$.* For simplicity, Na^+ influx is shown as a linear function of $[Na^+]_i$ in a normal situation and during increased influx e.g. due to increased leak (higher influx). The Na^+ transport rate by the Na^+/K^+-ATPase is shown as sigmoid saturation curves (Hill-curves) with a normal affinity for Na^+ (full line) and increased affinity for Na^+ (dotted line). Points of intersection reflect steady state $[Na^+]_i$.

In recent years, evidence has accumulated for the existence of a subsarcolemmal space where $[Na^+]_{ss}$ can vary largely from bulk $[Na^+]_i$ [144]. However very little is known about how the regulation of $[Na^+]_{ss}$ occurs, and how $[Na^+]_{ss}$ influences Na^+/Ca^{2+}-exchanger activity. A possibility is that the subsarcolemmal space for Na^+ may actually be small, separate "pockets" that are localized to various regions of the membrane. In this concept, localization of proteins is of crucial importance. Proteins located in separate "pockets" will not "see" the same $[Na^+]_i$, but they will if they are located within the same "pocket". This might also underlie the existence of different isoforms of some proteins. For example, two isoforms are expressed for the Na^+/K^+-ATPase (see below). Thus, they might play different roles based on their localisation in a specific "pocket". For example, a study of mice with genetically altered amounts of α_1- and α_2-isoforms suggest a selective involvement of the α_2-isoform in Ca^{2+} regulation, whereas the α_1-isoform plays a more "housekeeping" role [66]. A recent study, similar to Paper 2 in this thesis, provided further evidence for such role for the α_2-isoform [11].

Increased $[Na^+]_i$ is a common finding in models of heart failure. An increase of 3 mM was recently found in a rabbit pressure and volume overload heart failure model [6;40]. Increased $[Na^+]_i$ will alter the driving force for the Na^+/Ca^{2+}-exchanger, favouring Ca^{2+} influx (trigger Ca^{2+}) through reverse mode Na^+/Ca^{2+}-exchange. Increased reverse mode Na^+/Ca^{2+}-exchange was recently shown in a model of heart failure [93]. Increased $[Na^+]_i$ will also reduce Ca^{2+} efflux through forward mode during relaxation, thus contributing to the slow relaxation observed in heart failure. As a consequence, less Ca^{2+} will be transported out of the cardiomyocyte. This will tend to increase SR Ca^{2+} load, which might actually compensate for the reduced SR Ca^{2+} load commonly observed in heart failure. This could offset the depression of Ca^{2+}

transients and contractile function observed in heart failure [12]. Clearly, it is important to examine how local [Na^+] is regulated in the subsarcolemmal space, and how this regulates the Na^+/Ca^{2+}-exchanger.

3.3. The Na^+/K^+-ATPase

The Na^+/K^+-ATPase was first discovered in 1957 when J. C. Skou suggested that the transport of Na^+ and K^+ over the sarcolemma was coupled to a Na^+ and K^+ activated ATPase [125]. 40 years later, the Nobel Prize in Chemistry was awarded for this discovery. An increasing number of studies have focussed on the role for the Na^+/K^+-ATPase in the heart. However, few studies have assessed the functional role of different isoforms of the Na^+/K^+-ATPase.

3.3.1. Structure and isoforms

A functional Na^+/K^+-ATPase is assembled by at least one 110 kDa catalytic α-subunit and one 35-55 kDa glycosylated β-subunit. The α-subunit is composed of ~1000 amino acid residues and spans the sarcolemma 10 times. It contains binding sites for Na^+, K^+, ATP and cardiac glycosides and phosphorylation sites [105]. Thus, the function of the Na^+/K^+-ATPase depends mainly on the α-isoform present. Three different α-isoforms, encoded by three distinct genes, have been identified in rat and human hearts, α_1, α_2, and α_3 [80;112;145]. The α_1-isoform is the predominant isoform whereas the α_2 and α_3 isoforms are expressed at lower levels [34]. The α_3-isoform is expressed in moderate amounts in neonatal rat ventricle, but this expression declines after birth whereas the expression of α_2-isoform increases [34]. The α_3-isoform is barely detectable in protein homogenates from adult rat left ventricle [112].

The β-subunit is a single span protein which contains ~300 amino acid residues [52]. The C-terminus of the protein interacts with the α-subunit on the extracellular side [47]. Three isoforms have been identified (β_1, β_2, β_3), but only β_1 and β_2 are found in heart tissue. However, the β_2-isoform is expressed at a very low level in both human [145] and rat hearts [118]. A recent study suggested that in rats, β_1-isoforms associate with α_1- and α_2-isoforms, whereas β_2-isoforms, although expressed at a very low level, associate with α_3-isoforms in the intercalated discs [57]. The physiological role of the β–subunit is still unclear, but it has been shown that it is indispensable for the assembly of the Na^+/K^+-ATPase [21;67], and that it regulates the level of Na^+/K^+-ATPase inserted in the cell membrane [84]. A role for the β-isoform in ATP hydrolysis, ion transport and binding of inhibitors such as ouabain has also recently been suggested [105].

During translocation of ions, the Na^+/K^+-ATPase cycle between two conformational states, E_1 and E_2. The ion binding site is accessible from only one side at any given time and a varying affinity for Na^+ and K^+ in each conformational state provide for which ion that binds to the ionophore. Thus, the Na^+/K^+-ATPase translocates 3 Na^+ out of the cell, and then 2 K^+ into the cell using the metabolic energy of one ATP molecule. One net charge is moved out of the cell for each cycle, generating a small, but measurable current. The Na^+/K^+-ATPase pumps Na^+ and K^+ against their concentration gradients and is responsible for establishing and maintaining the electrochemical gradients for Na^+ and K^+ in cardiomyocytes. The density of Na^+/K^+-ATPase is several orders of magnitude higher than for Na^+ channels (1200 vs. 3/μm^2, [12]), but the turnover rate is ~4 orders of magnitude slower (80-100/s, [52]). However, the expression of the α-isoforms of the Na^+/K^+-ATPase is

heterogeneous in the sarcolemma. Despite several recent advances, a clear picture of this heterogeneous distribution has not yet been established.

3.3.2. Regulation

The Na^+/K^+-ATPase is regulated at multiple levels. It is dependent on intracellular Na^+, extracellular K^+, ATP, and can be modulated by cardiac glycosides. Further, it has recently become evident that important modulation of Na^+/K^+-ATPase activity in cardiomyocytes occurs through regulation by phospholemman, a transmembrane protein.

Na^+ and K^+ dependence

The Na^+/K^+-ATPase is regulated by its substrates: intracellular Na^+ and extracellular K^+. The half-maximal activation ($K_{0.5}$) for Na^+/K^+-ATPase is reached at a $[K^+]_o$ of ~1.5 mM [89], so at a normal $[K^+]_o$ of 4-5 mM, the Na^+/K^+-ATPase should be ~80% saturated with respect to $[K^+]_o$. This means that small variations in $[K^+]_o$ have little effect on Na^+/K^+-ATPase activity. Binding of K^+ to the Na^+/K^+-ATPase is considered voltage-dependent since K^+ must pass an access channel within the electrical field across the sarcolemma to reach its binding site [52]. Under experimental conditions, when K^+ is low and at positive potentials, the rate of the Na^+/K^+-ATPase can be reduced by electrostatic inhibition of K^+ binding [100]. This can cause a negative slope of the current-voltage relationship of the Na^+/K^+-ATPase at positive potentials.

The $K_{0.5}$ value for half-maximal Na^+/K^+-ATPase activation by $[Na^+]_i$ has been difficult to establish due to varying experimental conditions in different studies. This variation can be ascribed to: 1, differences between species. 2, difficulties of clamping the $[Na^+]_i$ by patch pipettes because of restricted diffusion in the subsarcolemmal space. 3, the fact that intracellular K^+ are competitive inhibitors of

Na^+ at intracellular Na^+ binding sites, and $[K^+]_i$ varies in different studies. However, at physiological conditions, the $K_{0.5}$ for Na^+ may be in the range of 10-20 mM [52;111;113] (but see also below about phospholemman). This is close to the normal resting $[Na^+]_i$ so small variations in $[Na^+]_i$ can have dramatic effects on the Na^+/K^+-ATPase activity. The affinity of the Na^+/K^+-ATPase to intracellular Na^+ increases during depolarization. This might play an important role during depolarization of the cardiomyocyte so that Na^+/K^+-ATPases pump out Na^+ that came in through Na^+ channels. Moreover, a low Na^+/K^+-ATPase activity during the resting potential might allow for accumulation of $[Na^+]_i$ during the resting potential. Thus, the Na^+/Ca^{2+}-exchanger is allowed to reverse during the upstroke of the action potential, bringing Ca^{2+} into the cell to trigger Ca^{2+} release.

Catalytic modulation

The Na^+/K^+-ATPase hydrolyses a molecule of ATP for each cycle. Various $K_{0.5}$ values for half-maximal Na^+/K^+-ATPase activation by ATP have been reported, ranging from 80 to 800 μM depending on species, isoforms and methods used [52;135]. However, physiological concentrations of ATP are in the order of 5-10 mM, and thus saturating for the activity of the Na^+/K^+-ATPase. Only under certain pathophysiological conditions, such as ischemia, a severe decrease in [ATP] in combination with an increase in [ADP], $[P_i]$ and $[H^+]$ could reduce Na^+/K^+-ATPase activity [110].

Cardiac glycosides

Cardiac glycosides, such as ouabain and digitalis, are potent inhibitors of Na^+/K^+-ATPase activity. They bind reversibly to the extracellular side of the α-isoform of the

Na$^+$/K$^+$-ATPase and inhibit ATP hydrolysis, and thus ion transport. The sensitivity of

the Na$^+$/K$^+$-ATPase for ouabain varies largely depending on species and α-isoform.

In rats, mice and guinea pigs, the Na$^+$/K$^+$-ATPase current can be separated into a

ouabain-sensitive and –insensitive component [65], although this separation is

discussable in the guinea pig. These components correspond to the α_2- and α_1-

isoforms, respectively. The affinity for ouabain has been assessed in several species.

Some results from intact ventricular cardiomyocytes are summarized in Table 1.

Table 1: Comparison of affinities for ouabain of Na$^+$/K$^+$-ATPase isoforms in different studies of intact ventricular cardiomyocytes

Preparation	Ouabain affinity α_1 $K_{0.5}$ (μM)	Ouabain affinity α_2 $K_{0.5}$ (μM)	[Na$^+$]$_i$ (mM)	[K$^+$]$_o$ (mM)	Ref
Rat ventricular myocytes	141	0.38	100	4	[41]
Rat ventricular myocytes	43.4	19.9 10^{-3}	50	5.4	[133]
Rat ventricular myocytes	43	1	15 or 100	15	[65]
Rat ventricular myocytes	33.9	1.1	15 or 100	15	[10]
Mouse ventricular myocytes	105	0.3	50	5	[11]
Guinea pig ventricular myocytes	72 (DHO)	0.75 (DHO)	50 or 10	1 or 4.6	[50]
Guinea pig ventricular myocytes	64.5 (DHO)	0.05 (DHO)	30	5.4	[87]

$K_{0.5}$: concentration for half maximal activation, DHO: dihydroouabain

As evident from the table, the affinity for ouabain of the Na$^+$/K$^+$-ATPase vary between

studies, especially for the α_2-isoform. An obvious explanation for this is the apparent

difference between species, but it could also be due to different experimental

conditions. In particular, it has been showed that association rate constants of

ouabain on the Na$^+$/K$^+$-ATPase are dependent on [K$^+$]$_o$ and [Na$^+$]$_i$ [19]. Extracellular K$^+$

is antagonising the inhibition of I$_{NKA}$ by ouabain, whereas augmented [Na$^+$]$_i$ increases

the probability for the Na$^+$/K$^+$-ATPase to take on a conformation that binds ouabain.

However, the latter effect was most prominent between 5 and 15 mM of Na$^+$ [52]. Thus,

it seems important to determine the affinities for ouabain for each experimental condition used.

The inhibitory effect of the Na^+/K^+-ATPase by cardiac steroids is clinically relevant. Digitalis has been used in the treatment of heart failure, or "dropsy", for more than 200 years [151]. However, the mechanism of action is still not completely understood. The beneficial effect of Na^+/K^+-ATPase inhibition by digitalis is ascribed to the positive inotropic effect resulting from accumulation of cellular Ca^{2+}. However, toxic effects are common, and might occur when cellular $[Ca^{2+}]_i$ increase to excessive levels, causing arrhythmias and potentially sudden death. In a randomized controlled Digitalis Intervention Group (DIG) trial, no decrease in overall mortality was observed after treatment with digoxin, although hospitalizations were reduced in patients with an ejection fraction lower than 45% [134]. However, a *post hoc* analysis of the DIG trial concluded that low serum concentrations of digoxin (0.5-0.9 ng/ml) reduced both mortality and hospitalizations in all heart failure patients [1]. A possible explanation could be that at low doses, digoxin affects a subgroup of Na^+/K^+-ATPases giving a beneficial effect. At higher doses this beneficial effect could be blunted by the inhibition of other Na^+/K^+-ATPases responsible for toxic effects. However, this explanation remains speculative until experimental data becomes available.

Phospholemman

In recent years, it has become evident that the Na^+/K^+-ATPase can be regulated by an associated, phosphorylable protein called phospholemman. Phospholemman is a small (72 amino acids) single span protein which belongs to a family of proteins called FXYD proteins, characterized by the presence of a conserved amino acid motif (Pro-Phe-X-Tyr-Asp). Phospholemman, which is also called FXYD-1, is mainly

expressed in the heart, skeletal muscle and liver [51]. It has been shown by co-transfection experiments in oocytes that phospholemman associates with Na^+/K^+-ATPase α_1- and α_2-isoforms [37]. However, in native membranes (bovine sarcolemma), the efficiency of co-immunoprecipitation with phospholemman was higher for the α_1-isoform than for the α_2-isoform. Moreover, in a recent study with immunoprecipitation and immunofluorescence experiments, phospholemman was found associated with the α_1-isoform, but not the α_2-isoform [119]. Binding of phospholemman to the Na^+/K^+-ATPase caused a decrease in the apparent affinity for $[Na^+]_i$ by nearly 2-fold, and also a small decrease in the affinity for $[K^+]_o$ [37]. This inhibitory role of phospholemman was further supported by using phospholemman knock-out mice [42]. Here the authors demonstrated that inhibition of the Na^+/K^+-ATPase by phospholemman mainly occurs through reducing the affinity for $[Na^+]_i$. However, the inhibitory effect of phospholemman can be relieved through phosphorylation by both α- and β-adrenergic agonists. Phosphorylation of phospholemman occurs at two sites: Ser 63 and Ser 68 in the C-terminal region. Ser 63 can be phosphorylated by PKC, and Ser 68 can be phosphorylated by both PKC and PKA [13]. Phosphorylation changes the interaction between phospholemman and the Na^+/K^+-ATPase, but does not lead to dissociation of the two proteins [13]. However, the complete picture of how the regulation of Na^+/K^+-ATPase activity by phospholemman occurs is yet to be determined.

3.3.3. Alterations in Na^+/K^+-ATPase activity heart failure

In various models of heart failure, decreased levels of Na^+/K^+-ATPase protein have been reported. In human heart failure there was an isoform specific decrease in the expression of $\alpha_1\beta_1$ and $\alpha_3\beta_1$ heterodimers in homogenates from left ventricle tissue.

The protein level of α_2-isoforms was unaltered [108]. Another study revealed a trend for overall decrease of Na^+/K^+-ATPase protein in human myocyte fractions (24%) [25], although this reduction did not reach statistical significance. In rabbit heart failure, a 36% overall decrease in Na^+/K^+-ATPase protein was observed using a pan-specific antibody [25], and expression of all isoforms was lower in myocytes (30%, 17%, and 58% for α_1, α_2 and α_3, respectively). In protein homogenates from a rat post infarction CHF model, expression of α_1-isoforms was unaltered, whereas α_2-isoforms were lowered by 55% and α_3-isoforms were induced from a barely detectable level [112]. However, as pointed out in another study, the increase in α_3-isoform is likely to occur in the non-myocyte fraction [25]. The overall reduction in Na^+/K^+-ATPase protein could be an explanation for the increased $[Na^+]_i$ observed during heart failure [40;95]. A study concluded that the Na^+/K^+-ATPase pump capacity was reduced in CHF because of cardiomyocyte hypertrophy, so that a same number of pumps could not readily control $[Na^+]_i$ [112]. However, unaltered Na^+/K^+-ATPase capacity in heart failure has also been shown [40]. Here, the authors suggested that the increase in $[Na^+]_i$ was due to increased influx of Na^+ through slowly inactivating Na^+ channels. Another study explained the increased $[Na^+]_i$ by an upregulation of the Na^+/H^+-exchanger [6]. An explanation for reduced expression of Na^+/K^+-ATPase does not necessarily cause a decrease in Na^+/K^+-ATPase function came with the discovery that the expression of phospholemman is also reduced in heart failure [25]. In combination with increased phosphorylation of phospholemman in heart failure, the inhibition of Na^+/K^+-ATPase by phospholemman is blunted, resulting in unaltered Na^+/K^+-ATPase function [13].

Despite these recent data, alterations in Na^+/K^+-ATPase function during heart failure are not fully understood. Clearly, Na^+/K^+-ATPases as well as other proteins involved in excitation-contraction-relaxation cycle "see" the ion concentrations in their

immediate vicinity. These do not necessarily reflect the global ion concentration in the cytosol. Alterations in local "pockets" of ions could occur during heart failure, causing alterations in ion homeostasis. Such alterations could be due to remodelling of the cardiomyocyte structure, for example in the t-tubules.

3.4. T-tubules in cardiomyocytes

A particular feature of the sarcolemma in ventricular cardiomyocytes is the presence of transverse tubules (t-tubules). T-tubules are invaginations of the cell membrane which form a tortuous network in cardiomyocytes. Recent research has demonstrated that their structure and function plays an important role for cardiomyocyte function.

3.4.1. Structure of the t-tubules

T-tubules owe their name to early studies showing that they appear as *transverse* segments at intervals of ~1.8 µm near the Z lines in cardiomyocytes [91]. The Z lines are dark bands seen in the microscope which correspond to the protein structures between sarcomeres (Z is from the German *zwischen* which means *between*). Since then, it has become apparent that t-tubules also occur in the longitudinal direction [127]. A more recent study constructed a three-dimensional model of the t-tubules based on stacks of confocal images of live rat cardiomyocytes [126]. The authors quantified the t-tubules which occur near the Z line to ~60% of all the t-tubules, whereas the remaining ~40% occur between the Z lines. It has now become clear that the t-tubules form a complex network containing branching tubules in both transverse and longitudinal directions [30].

The diameter of the t-tubules varies from 20 to 450 nm, but more than 50% have a diameter of 180-280 nm [126]. Their total volume has been estimated to 0.8-3.6% of the cardiomyocyte volume (see [12] for Refs). Estimates of the fraction of

sarcolemma in the t-tubules (vs. surface membrane) range from 21-64% [12]. However, experiments involving an osmotic shock (see 5.4 Detubulation) to uncouple the t-tubules from the surface membrane show a ~30% decrease in cell capacitance upon detubulation [31]. Although this large variability in the estimates of t-tubule morphology might reflect methodological aspects, it might also be due to the large plasticity of the t-tubules. Indeed, t-tubules are absent in the neonatal heart [35;56], and disappear when cells are kept in culture [76;78]. Moreover, reports show remodelled t-tubule structures during heart failure (see below). How the t-tubules maintain their morphology, or fail to do so, is still uncertain. A working hypothesis could be that the t-tubules need a scaffold made of proteins in the cytoskeleton and that alterations in the cytoskeleton will cause t-tubule remodelling. Further studies are necessary to examine this possibility.

3.4.2. Functional role of t-tubules

By providing proximity between the sarcolemma, the SR and the contractile apparatus, the t-tubules predispose for an efficient coupling of electrical excitation of the cell membrane to Ca^{2+} release and contraction. Several studies have shown that the density of key proteins in excitation-contraction-relaxation coupling appears to be higher in the t-tubules than in the surface membrane [31]: For example, L-type Ca^{2+} channel density is 6 times higher in the t-tubules than in the surface membrane [29]. By this specialized subcellular localization, the L-type Ca^{2+} channels in the t-tubule membrane face RyR in the SR membrane in specialized microdomains (dyads) allowing efficient gain of the Ca^{2+} induced Ca^{2+} release. The importance of this coupling has been demonstrated in experiments with cardiomyocytes lacking t-tubules (detubulated [28;153], cultured [78] and atrial [22]). Here $[Ca^{2+}]$ initially rises near the surface membrane of the cell, and then diffuses into the cell interior, causing

dyssynchronous Ca^{2+} release. Thus, adequate subcellular localization of the L-type Ca^{2+} channel in the t-tubule membrane is necessary to allow synchronous Ca^{2+} release throughout the whole cardiomyocyte.

Likewise, the densities of Na^+/Ca^{2+}-exchangers and Na^+/K^+-ATPases have been found 3 times higher in the t-tubules [39]. Thus, co-localization of these proteins is likely, and they are possibly interacting to regulate $[Na^+]_{ss}$. However, how the t-tubules contribute to a specialized microdomain for Na^+ which is linked to Ca^{2+} handling is less well established. Moreover, alterations in t-tubule structure and function might disturb the co-localization of proteins and thus the microdomains for both Na^+ and Ca^{2+}, leading to mismanagement of Ca^{2+} handling. The results presented in this thesis shed new light into these issues.

To summarize, the t-tubules allows an efficient, synchronous Ca^{2+} handling in the cardiomyocyte during the cardiac cycle without the need for individual ions to travel very far. This is important since diffusion of solutes appears to be slow in cardiomyocytes. In the same way, diffusion of ions could be slow in the t-tubules. Thus, the t-tubules might constitute a specialized compartment where ions can accumulate and deplete during the cardiac cycle. Such accumulations and depletions of ions may influence the spread of the action potential and proteins important in the excitation-contraction-relaxation coupling.

3.4.3. Changes in t-tubules during heart failure

Several recent reports show alterations in the t-tubular network in models of heart failure. He *et al*. [59] showed that the density of both transverse tubules and L-type Ca^{2+} channels was reduced in a canine model of tachycardia-induced heart failure. Other studies have demonstrated increased spatial dispersion of the t-tubules leading to Ca^{2+} instability in heart failure [32;79;128]. However, the link between

alterations in the t-tubule structure and alterations in excitation-contraction-relaxation coupling is still missing [31]. Whether it is the altered geometry of t-tubules in itself, or if it is the altered localisation of key proteins which plays the most important part in Ca^{2+} mismanagement is a matter of debate. It might be that altered geometry disrupts specialized microdomains, and that this in turn leads to alterations in ion handling proteins.

4. MAIN AIMS

Main aims:

The main aims of the present thesis were to examine to which extent diffusion of ions is restricted in the t-tubules and in the intracellular compartment, and to investigate to which extent restricted diffusion of ions affects cardiac excitation-contraction coupling.

Specific aims:

Explore the role of the t-tubules as a separate functional compartment by examining whether diffusion of K^+ is restricted in the t-tubules.

Investigate the role of the Na^+/K^+-ATPase α_2-isoform in the regulation of Na^+/Ca^{2+}-exchanger activity.

Determine the expression pattern of the Na^+/K^+-ATPase α_2-isoform in heart failure and to identify the functional consequences of Na^+/K^+-ATPase α_2-isoform downregulation.

5. METHODS

5.1. Animal model

Hearts from male Wistar Hannover rats (Møllegaard Breeding and Research Center, Denmark) weighing ~300g were used in all animal experiments in this thesis. This is an outbred strain which has a larger biological variability than inbred strains. The choice of this animal model was based on ethical and economical considerations. Moreover, our laboratory has extensive experience with this strain. However, this strain also exhibits advantages for studying the problems addressed in this thesis. First; it has been reported that the rat has a more extensive t-tubule network than other species [30]. With the reservation that this variation might be due to methodological rather than biological differences, it makes the rat a very suitable model for studying the questions addressed in Paper 1. Second; in contrast to other species, like humans [146], the various Na^+/K^+-ATPase isoforms in rat cardiomyocytes have different affinities for ouabain. This property was exploited in Papers 2 and 3.

However, some important differences between cardiomyocytes from rats and humans exist, restricting extrapolations of the results to humans [12;58]: 1; the action potential of rat cardiomyocytes is much shorter and lacks a prominent plateau phase. 2; the Na^+/Ca^{2+}-exchanger is relatively less important for Ca^{2+} removal from the

cytosol in rats than in humans. 3; the force frequency relationship is negative in rats, but positive in humans. 4; the resting rat heart rate is five times that of humans. 5; the levels of $[Na^+]_i$ are higher in the rat. Despite these differences, the results presented in this thesis can increase our understanding of cardiomyocyte function. Further, the results can be compared with results obtained in rats in other laboratories around the world.

In Paper 3, we have used a post infarction CHF model, mimicking human heart failure resulting from coronary artery occlusion. This model has been described in detail elsewhere [138]. Also here, one should be careful to extrapolate our results to humans. Several differences exist between experimental heart failure in rats, and chronic heart failure in humans: 1; in the rats the coronary artery occlusion was induced in healthy young individuals, whereas myocardial infarctions in humans usually occur in the elder. 2; CHF in humans usually develops over years, whereas in our experimental model CHF was studied after 6 weeks. 3; CHF in humans is usually multifactorial [58].

5.2. Hemodynamics

Not all the animals with induced myocardial infarction developed CHF. As described by Sjaastad *et al.* [123], only rats with a left ventricular end diastolic pressure (LVEDP) ≥ 15 mmHg and increased lung weight were considered to have CHF. To verify that animals included in Paper 3 developed CHF, we catheterized the aorta to measure blood pressure and LVEDP using a 2F (0.67 mm) microtip pressure catheter (model SPR-407, Millar Instruments Inc., Houston, TX). This was also done in the Sham group for comparison.

5.3. Cell isolation

Cell isolation is a crucial point in our experimental procedures. Bad cell quality is often associated with a low yield, cardiomyocyte instability and difficulties in obtaining a gigaohm seal (see 5.5 Electrophysiological methods). This often results in no, or at best poor quality recordings. Even though cell isolation procedures are so important, little space is left in published papers for this topic. Therefore, a short description of the procedure is presented, and important aspects are commented.

Isolated cardiac ventricular myocytes were prepared using an enzymatic perfusion method. After a thoracotomy, heparin was injected through vena cava inferior before excising the heart in order to avoid coagulation in the coronary arteries. The aorta of the excised heart was cannulated above the aortic valve and the coronary arteries were perfused with a preoxygenated solution at 37°C for 1-2 minutes. An enzyme solution containing 1 g.l^{-1} collagenase type II (Worthington) was then perfused until the aortic valve was digested, as attested by the increased outflow of perfusate. We experienced that inadequate perfusion is one of the main reasons for obtaining a low yield of live cardiomyocytes at the end of the isolation procedure. This can probably be explained by poor oxygenation of the tissue and consequent cell death. Also, poor perfusion means that the enzyme is not delivered to all parts of the heart, leading to inefficient digestion of the tissue. Another critical factor for obtaining good quality cardiomyocytes is the collagenase. Several collagenases exist, but the type II from Worthington is recommended for digestion of heart tissue. However, a lot-to-lot variation is typical of such crude enzyme preparations, and makes it important to pre-test a particular lot of enzyme for experiments. The reason is that crude collagenase preparations contain not only collagenases but also a sulfhydryl protease, clostripain, a trypsin-like enzyme, and an

aminopeptidase. This combination of collagenolytic and proteolytic activities is effective at breaking down intercellular matrices, the essential part of tissue dissociation. It is the combined action of these enzymes that varies between lots. Empirically, we found that lots with a collagenase activity close to 200 U/ml gave a good yield.

The cell isolation procedure potentially affects the cardiomyocytes studied in many ways. First, the collagenase could affect membrane proteins important to cardiomyocyte function, especially because of protease activity. Therefore, we did not switch between lots within comparable series of experiments. Second, to make sure that only cardiomyocytes from the left ventricle and septum were studied, the atria and the right ventricle were cut off early in the isolation procedure. Third, in hearts from CHF rats, a large part of the left ventricular free wall was infracted. The infarct area and border zone were cut off, leaving mainly the septum for experiments. Thus, our cell experiments in CHF are mostly representative of the septum. Fourth, regional differences exist within the left ventricle. However, in paired experiments, this would not cause a problem.

5.4. Detubulation

The detubulation technique of live cardiomyocytes using an osmotic shock was developed in Clive Orchards lab in 1999 [68]. Before this technique became available, t-tubules were studied mainly using immunohistochemical studies, telling tales of protein distribution, but not of protein function in the t-tubules. Detubulation has been used in several studies providing important information about t-tubule function in cardiomyocytes. However, some concerns can be raised concerning this technique. Formamide ($HCONH_2$) which is used to induce the osmotic shock, is an amide analogous to urea ($CO(NH_2)_2$), and can potentially have direct effects on cardiac cells

due to its properties as a denaturant. Thus, disruption of protein-protein interactions as well as re-distribution of membrane proteins could occur. These problems were addressed in a paper by Brette *et al.* [28]. The authors found that formamide treatment of atrial cells, which lack t-tubules, did not change cell capacitance, Ca^{2+} current amplitude, action potential configuration, the Ca^{2+} transient or the response of the Ca^{2+} transient to isoprenaline. Although these results did not prove the absence of possible protein re-distribution, they suggest that this is unlikely to occur. However, there is no direct proof in the literature today that Na^+/K^+-ATPases are not re-distributed due to the formamide treatment. Therefore it is important to emphasize that in Papers 2 and 3, all conclusions as to the functional crosstalk with the Na^+/Ca^{2+}-exchanger and consequences for $[Na^+]_i$, contractions and Ca^{2+}-transients are independent of the detubulation experiments. Hence we did not interpret the detubulation experiments extensively, we only concluded as to whether the isoforms of the Na^+/K^+-ATPase were located in the surface membrane or in the t-tubules.

5.5. Electrophysiological methods

In all the studies in this thesis, we used a complex method generally known as the patch clamp technique to measure membrane potentials and currents across cardiomyocyte membranes. Although the term "patch clamp" originally refers to experiments studying individual ion channels in "patches" of cell membrane, it is also often referred to for whole-cell recordings, as in Papers 1-3. Patch clamping is a complicated technique, requiring many considerations. Here, only some of the principles will be presented, and implications for our experiments will be commented.

For the patch clamp experiments, we used a glass pipette which constituted an electrode when filled with an electrolyte solution mimicking that of the cytosol. This electrode was pressed against the cell surface. When suction was applied in the

glass pipette, the cell membrane formed a tight seal with the electrode, a so-called "gigaohm" seal, since the electrical resistance of that seal is high. To access the whole cell, the membrane patch defined by the pipette tip was ruptured, establishing direct contact between the solution in the pipette and the cytosol. The mixing of these two solutions, also referred to as dialysis of the cell, depends on the size of the pipette tip. A bigger tip means lower electrical resistance of the electrode, and more efficient dialysis. This was a crucial point in Papers 2 and 3, where we "clamped" the intracellular solutions, in particular the $[Na^+]_i$.

In all studies in this thesis, we used continuous single electrode voltage clamp (cSEVC). Here, a single electrode was used to clamp voltage and to pass current simultaneously. Since current flowing through a non-zero resistance (the electrode) necessarily produces a voltage drop across that electrode, this can introduce a source of error in the voltage clamping. The measured voltage is actually the sum of the voltage across the pipette and the membrane potential (which is what we want to measure). To keep the voltage drop to a minimum, the series resistance of the pipette can be compensated by the amplifier. However, due to practical limitations, 100% compensation cannot be achieved. An alternative approach would be to use discontinuous single electrode voltage clamp (dSEVC). With that method, switching between passing of current and measuring of membrane potential would allow us to measure the true membrane potential, since almost no current is passed while measuring the membrane potential. Yet, dSEVC is also noisier, limiting its advantages in our experiments where currents are relatively small. Moreover, the advantages of dSEVC are particularly important in protocols where fast voltage steps are required. Measuring relatively slow changes in membrane potentials using cSEVC should not be a problem, since problems related to series resistance would

be negligible. The change in voltage in our experiments was several orders of magnitude slower than that allowed by the amplifier. For example, in Paper 1, the membrane potential changed with a maximum rate in the order of ~0.02 mV/ms, and the junction potential in the order of ~0.2 mV/ms. This is several orders of magnitude slower than the rapid depolarisation of an action potential (in the order of ~200 mV/ms). This was further confirmed by the similar kinetic parameters recorded both in voltage and current clamp in Paper 1. Also, the pipette resistance in our experiments was so low that errors due to series resistance were kept to a minimum.

5.6. Contraction measurements

In Paper 2 and 3, contractions were recorded in field stimulated cardiomyocytes using a video-edge detection system. Fractional shortening is typically reduced in post infarction CHF [121;123], but has also been reported to be unchanged [3;99]. However, in Paper 3 we observed an increased fractional shortening of cardiomyocytes from CHF hearts. This discrepancy requires some considerations.

First; in our experiments, cardiomyocytes were plated on laminin-coated coverslips, and were allowed to contract with little resistance against movement. This unloaded shortening is quite different from the *in vivo* situation where cardiomyocytes are connected to each other and therefore must contract against an afterload. Thus, results obtained using this method might not be representative for the *in vivo* situation. Second; the cardiomyocytes were field stimulated by passing current in the cell bath. This is quite different from the *in vivo* situation where contractions are initiated by an action potential spreading form cell to cell. It has been reported that field stimulated cell contraction is independent of sodium currents, but is triggered by a combination of L-type Ca^{2+} current and reverse mode Na^+/Ca^{2+}-exchange [24]. Third; our experiments were performed at 1 Hz. This is slow compared to physiologic heart

rates in rats. It was shown in human heart failure that reduced force of contraction was more apparent at physiological heart rates than at low frequencies [94]. Moreover, because contractile properties can be similar in normal and failing muscles under basal conditions, but become apparent during stress, contractile dysfunction in CHF should be studies at variable rates of stimulation and during stimulation by inotropic drugs [61]. However, in Paper 3 we studied the downregulation of the α_2-isoform and consequences for regulation of contractility through the Na^+/Ca^{2+}-exchanger. Thus, a description of the altered contractile properties in CHF was beyond the scope of that paper.

5.7. Immunocytochemistry

In Paper 2 and 3 we used antibodies to study the subcellular distribution of Na^+/K^+-ATPase α-isoforms. In this method, fixed, permeabilized cardiomyocytes are incubated with an antibody against the α_1- and α_2-isoforms of the Na^+/K^+-ATPase. Then fluorochrome labelled secondary antibodies against the primary antibodies are used to visualize the localisation of the Na^+/K^+-ATPases. This permits signal amplification because several secondary antibodies binds to different antigenic sites on the primary antibody.

Several steps in the protocol are critical to obtain good quality immunostains. First of all, the specificity of the immunostain is dependent on the specificity of the antibody. Further, to reduce unwanted unspecific binding, the cells are incubated in goat serum to reduce the number of epitopes available for unspecific binding. The fixation and permeabilization conditions can also influence the integrity and availability of epitopes. Therefore optimizations of labelling protocols are required. This optimisation is often based on experience and empirical trial and failure. Thus,

to avoid variations in the images due to differences in labelling protocols, conditions were set equal in comparable series. Moreover, in experiments comparing Sham and CHF, labelling was performed in parallel the same day.

During sampling of images on the confocal microscope, optical distortion occurs due to imperfections in the optical pathway. In our experiments, this "noise" was reduced by blind deconvolution using commercially available software algorithms (Huygens software, SVI, The Netherlands). This method deduces a point spread function, a mathematical function that describes the distortion which occurs during recording (convolution) of the image. Knowing this point spread function, the near original image can be computed. Deconvolution of images preserves quantitative relationships, and may therefore also be used in preparation for quantitative image analysis [131]. Nevertheless, the optical resolution of confocal images still represents a limitation for determining close co-localization of molecules. Hence, this method is primarily useful for determining in which part of the cell the target protein is located (e.g. in our case the t-tubules vs. the surface membrane).

5.8. Fluorescence

In Paper 1, 2 and 3 we have used fluorescent probes to visualize the sarcolemma, Ca^{2+} transients and intracellular Na^+ concentrations.

The sarcolemma was visualized using di-8-ANEPPS (Invitrogen) which is a membrane binding molecule. It is less susceptible to internalization than other similar dyes, so labelling of intracellular membranes such as sarcoplasmic reticulum or mitochondria membranes is limited.

The rise and fall of the cytosolic Ca^{2+} level was visualized using fluo-4 (Invitrogen). Upon excitation by light of a specific wavelength, fluo-4 exhibits a rapid increase in fluorescence intensity when binding to Ca^{2+}. The fluo-4 was loaded into

the cells as an acetoxymethyl (AM) ester which can permeate cell membranes. In the
cytosol, unspecific esterase activity cleaves off lipophilic blocking groups, leaving a
charged form which leaks out of cells far more slowly than its parent compound.
However, leak of dye into intracellular compartments could occur, influencing the
recorded Ca^{2+} transient. In particular, leak into the mitochondria would cause a
problem since they constitute ~35% of the cell volume [12]. To avoid this, we loaded
the cells at room temperature, which slows the speed of loading and leaves time for
deesterification. The time of loading was also limited, although a minimum is required
to get enough dye into the cell. Further, this loading protocol was standardized and
used in comparable groups. An Argon laser was used to excite the fluorescent probe,
representing high excitation intensity. To limit cell damage, the laser power and
scanning time were reduced to a minimum.

The intracellular Na^+ concentration was measured using sodium-binding
benzofurzan isophthalate (SBFI, Invitrogen). Traditionally, this dye is used in a dual
excitation configuration, where the ratio of fluorescence intensities obtained by
exciting at 340 nm and 380 nm, while measuring emission at 500 nm is used to
determine the concentration of Na^+. However, binding of SBFI to intracellular proteins
cause a shift of excitation and emission spectra. This was assessed in a study by
Baartscheer *et al.* [4]. They demonstrated that SBFI used in dual emission mode
provides a more sensitive and more specific method to measure small changes of
$[Na^+]_i$ in single cardiomyocytes. Thus, in Paper 2, SBFI was excited at 340 nm, and
emission was collected at 410 nm and 590 nm. SBFI was loaded as an AM ester,
and measures similar to those described for fluo-4 were taken to avoid leak of SBFI
into intracellular compartments. Finally, the SBFI is a rather slow probe, and

therefore it was not possible to study transient changes in $[Na^+]_i$ during the cardiac cycle.

5.9. Western blot analysis

In Paper 2 and 3, we used Western blots to determine protein specificity and to measure protein levels in homogenates of left ventricle tissue. With this method, proteins are initially separated by sodium-dodecyl-sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) before they are blotted over to a membrane which is incubated with the primary antibody. The amount of antibody binding is determined by a secondary antibody labelled with horseradish peroxidase giving a luminescent product which can be detected using a light sensitive video camera. Western blot requires many methodological considerations. I will here only briefly point out some important points for the present thesis: 1, since the antibody-antigen affinity is specific to each antibody, the amount of protein loaded on the gel must be adjusted adequately for each antibody used. 2, the appearance of a single band indicates a high specificity of the antibody since the other proteins, which are present on the blot, are not labelled. 3, Western blot is a semi quantitative method, so it is not possible to calculate protein concentrations. However, the amount of protein can be compared in two groups, like we did in Paper 3. 4, due to large blot-to-blot variability, comparisons should always be made on the same blot. 5, considering the semi quantitative aspect, small changes in immunolabelling density should be interpreted with care.

6. RESULTS AND DISCUSSION

6.1. T -tubules constitute a functional compartment

Despite the rather large diameter of t-tubules, the diffusion of solutes into the t-tubule appears to be slow [23;117;132;154]. In Paper 1, we show that diffusion of K^+ in the lumen of t-tubules is slow, ~85 μm^2/s. For comparison, the diffusion of K^+ in water is about nine fold faster [81]. Thus, the t-tubules can act as compartments where K^+ can accumulate and become depleted during the cardiac cycle. The experimental implication of our results is that during rapid perfusion of cardiomyocytes, a complete switch of the extracellular solution, including the lumen of the t-tubules, requires minimum 1 s. In the intact heart however, such rapid changes are unlikely to occur. Nevertheless, during the cardiac cycle, both influx and efflux of ions occur. A slow diffusion of K^+ could lead to accumulation of K^+ in the t-tubules during exercise. The role of extracellular K^+ accumulation has been studied in skeletal muscle in which K^+ shifts can cause muscle fatigue through the effect on muscle excitability and action potentials [110]. However, in the heart, the K^+ balance appears to be much more efficiently controlled [110]. This is probably because the rise of $[Na^+]_i$, following for example an increase in heart rate, is sufficient to activate the Na^+/K^+-ATPase to completely compensate increased K^+ release. However, in heart failure where the

Na$^+$/K$^+$-ATPase capacity could be reduced, as shown in Paper 3, and also by another study [112], the slow diffusion of K$^+$ could have greater consequences. Increasing heart rate when the capacity of the Na$^+$/K$^+$-ATPase is reduced could lead to increased [K$^+$]$_o$. The membrane will depolarize by almost 18 mV for each doubling of [K$^+$]$_o$ [110]. Thus, the resting membrane potential is brought closer to threshold for triggering an action potential. However, as seen in skeletal muscle, the effects of changes in [K$^+$]$_o$ on electrical properties are complex, and there is not a simple linear relationship between [K$^+$]$_o$ and force development [110]. Thus, even if hyperkalemia is a common cause of cardiac arrhythmias [92], two factors cause hyperkalemia to actually reduce cardiomyocyte excitability: (1) An increased [K$^+$]$_o$ increases the conductance of I$_{K1}$. Increased conductance of I$_{K1}$ means that more inward Na$^+$ current is required to trigger an action potential. (2) The depolarized membrane potential increases the fraction of inactivated Na$^+$ channels [12]. Due to the complexity of this regulation, a rough and ready calculation on the effect of stimulation rate on membrane potential based on our diffusion data would be speculative.

As discussed in Paper 1, the causes for the restricted diffusion are uncertain, but probably results from the tortuous structure of the t-tubules and because of ion binding sites in the t-tubule membrane and glycocalyx. In Paper 3, we observed altered structure of the t-tubule network structure during heart failure. Although we did not determine whether ion binding sites were altered, there was a transition in the morphology of t-tubules. In particular, there was a lesser association between the SR membrane and the t-tubule membrane. We speculate that such spatial alterations could lead to disturbances in local microdomains on both sides of the t-tubule membrane. This could constitute a structural basis for alterations observed in heart failure. Functional changes commonly observed in heart failure can be mimicked in

experiments with detubulated cells. These changes include reduced and slowed Ca^{2+} transient, less synchronized SR Ca^{2+} release, decreased response of $I_{Ca,L}$ to β-adrenergic stimulation, and decreased contractile response to cardiac glycosides [31]. Thus, intact t-tubule function is of crucial importance for cardiac function.

6.2. Na^+/K^+-ATPase α_2-isoform is preferentially located in t-tubules

The function of t-tubules in providing proximity between proteins involved in excitation-contraction-relaxation coupling is illustrated by the fact that precise targeting of L-type Ca^{2+} channels so that they face RyRs in the couplon is indispensable for synchronous Ca^{2+} release [79;128]. In Paper 2 and 3, we observed that the α_2-isoform of the Na^+/K^+-ATPase was preferentially located in the t-tubules of cardiomyocytes whereas the α_1-isoform was more abundant in the surface sarcolemma. However, controversy exists. By immunohistochemistry, studies have shown uniform distribution of α_1- and α_2-isoforms in the surface membrane with little staining in the t-tubules (rat) [130], uniform distribution of α_2-isoform in the whole sarcolemma, but α_1-isoform mainly in t-tubules (rat) [85], α_1-isoforms predominantly in surface sarcolemma and α_2-isoforms mainly in the t-tubules (guinea-pig) [119]. However, in these studies, different antibodies were used and the discrepancies in these results probably reflect differences in antibody specificity. Further, different accessibility of epitopes might exist between the t-tubules and the surface sarcolemma. This stresses the need to confirm data by functional measurements. In Paper 2 and 3, we confirmed our immunocytochemistry data on the functional level by comparing currents recorded in control and detubulated cardiomyocytes. Our results were similar to those obtained recently by Berry *et al*. in mice [11]. They

concluded that the α_1-isoform was almost uniformly distributed between t-tubules and surface sarcolemma whereas the α_2-isoform was ~5 times more concentrated in the t-tubules. Further, it has previously been shown that the density of Na^+/Ca^{2+}-exchangers is higher in the t-tubules than in the surface sarcolemma [39;136]. Thus, colocalization of Na^+/Ca^{2+}-exchanger with α_2-isoforms seems likely, and a Na^+/Ca^{2+}-exchanger regulatory role for Na^+/K^+-ATPase α_2-isoform can be predicted.

6.3. Na^+/K^+-ATPase α_2-isoform regulates cardiac function

A main finding in Paper 2 and 3 is that the α_2-isoform normally constitutes only a small fraction (8-11%) of all the Na^+/K^+-ATPases in the cardiomyocyte. This is in line with other reports [11;75]. Yet, a substantial contractile effect was observed when the α_2-isoform was inhibited by a low dose of ouabain (0.3 μM). A positive inotropic effect resulting from inhibition of the Na^+/K^+-ATPase has been ascribed to an increase in intracellular $[Na^+]_i$, resulting in increased Ca^{2+} influx via the Na^+/Ca^{2+}-exchanger [7]. In Paper 2, we showed that both diastolic and systolic $[Ca^{2+}]_i$ increased during blockade of the α_2-isoform, but we did not detect any increase in bulk $[Na^+]_i$ with SBFI. However, with the method used, we could not isolate $[Na^+]$ in the subsarcolemmal space from bulk $[Na^+]$. Thus, a possible explanation is that $[Na^+]_i$ increased only in the subsarcolemmal space. By snap-freezing cardiomyocytes and measuring the total $[Na^+]$ in samples of small volumes at different sites in the cell, Wendt-Gallitelli *et al.* demonstrated that $[Na^+]$ can be substantially higher in spaces beneath the membrane than in the bulk cytosol [150]. Since the activity of the Na^+/Ca^{2+}-exchanger is controlled by $[Na^+]_i$, we measured I_{NCX} to assess subsarcolemmal $[Na^+]$. Abrupt activation of I_{NKA} led to a decrease in I_{NCX}, presumably due to local depletion of $[Na^+]_i$ in the vicinity of the Na^+/Ca^{2+}-exchanger. This decrease was smaller when

the α_2-isoform was inhibited by a low dose of ouabain, indicating that the α_2-isoform is necessary to modulate $[Na^+]_i$ close to the Na^+/Ca^{2+}-exchanger. Thus, the Na^+/K^+-ATPase α_2-isoform and the Na^+/Ca^{2+}-exchanger may be tightly coupled in local microdomains in the t-tubules of cardiomyocytes. Alterations in colocalization or protein levels within this microdomain might have profound consequences for cardiac function.

6.4. Downregulation of Na^+/K^+-ATPase α_2-isoform in heart failure impairs cardiac function

In a previous study from our laboratory, it was shown that the α_2-isoform was downregulated whereas the α_1-isoform remained unchanged in CHF [112]. It was also shown that α_3-isoform expression increased from a barely detectable level. However, these experiments were performed on homogenates from cardiac tissue, and it was not possible to separate cardiomyocytes from other cell types in the heart. More recently, it was shown in a rabbit aorta banding heart failure model that, although the α_3-isoform was increased in homogenates, it was actually reduced in isolated cardiomyocytes, suggesting that the increased expression of α_3-isoform occurs in non-myocytes [25]. In that study, the α_2-isoform was reduced in both homogenates and isolated cells. However, the functional consequences of a reduction in α_2-isoform have not been assessed. In Paper 3 we studied the consequences of downregulation of the α_2-isoform.

First, it was crucial to determine whether the α_2-isoform was functionally reduced in CHF. The inhibitory effect of the low dose of ouabain on I_{NKA} at a holding potential of -50 mV was reduced by 78% in CHF, suggesting that the α_2-isoform was

functionally reduced. This was in surprisingly good agreement with the protein levels measured by immunoblots in protein homogenates (74%). Further, cardiomyocyte contractions were measured at 1 Hz in field stimulated cardiomyocytes. Here, baseline contractions (fractional shortening) were actually larger in CHF than in Sham. This is in agreement with some studies showing that at low stimulation rates, the contractile defect in heart failure can be blunted [103]. An explanation for this was given in a recent study, where it was reported that increased baseline $[Na^+]_i$ was responsible for maintaining contractility at low heart rates in a feline model of hypertrophy (by increasing the SR Ca^{2+} load) [86]. In our study, we did not measure $[Na^+]_{ss}$ directly in intact cardiomyocytes. However, in dialyzed cells, when I_{NKA} was abruptly activated after a $[Na^+]_i$ loading period, I_{NCX} decreased in Sham, presumably because of local depletion of $[Na^+]_{ss}$. This decrease was smaller when the α_2-isoform was downregulated (CHF) or inhibited (low dose ouabain), indicating depletion of $[Na^+]_{ss}$ to a lesser extent in these cells. Thus, downregulation of the α_2-isoform could increase the $[Na^+]_{ss}$ in baseline conditions, and constitute a compensatory mechanism to counteract reduced SR Ca^{2+} load at low heart rates. Further studies are necessary to determine the effects of downregulated α_2-isoforms at higher heart rates.

7. MAIN CONCLUSION

The present thesis demonstrates that diffusion of ions is restricted in the t-tubules and in the subsarcolemmal space. A functional consequence is that ions can accumulate and become depleted in local pools without affecting bulk ion concentrations. Thus, precise targeting of proteins involved in the excitation-contraction-relaxation coupling to discrete regions of the sarcolemma is important for the regulation of cardiac function.

The conclusions for each specific aim are:

Conclusion 1

The diffusion rate for K^+ in t-tubules of rat cardiomyocytes is slow, ~85 μm^2/s. This means that K^+ ions, and probably other ions, can accumulate or deplete in discrete locations in the t-tubules during the cardiac cycle. This could play an important role for cardiomyocyte function by influencing the spread of the action potential, and by influencing membrane proteins involved in excitation-contraction-relaxation coupling.

Conclusion 2

The Na^+/K^+-ATPase α_2-isoform constitutes only ~11% of all the Na^+/K^+-ATPases in rat cardiomyocytes. However, by localisation in discrete locations in the t-tubules of rat cardiomyocytes, the α_2-isoform is efficiently coupled to the Na^+/Ca^{2+}-exchanger. This occurs through a subsarcolemmal local pool of Na^+, and constitutes an important regulatory mechanism of cardiomyocyte contractility.

Conclusion 3

Altered t-tubule network structure and downregulation of the α_2-isoform in a rat post infarction CHF model cause attenuated control of Na^+/Ca^{2+}-exchanger activity, presumably due to local alterations in $[Na^+]_i$. This indicates that the Na^+/K^+-ATPase α_2-isoform is necessary to modulate $[Na^+]_i$ close to the Na^+/Ca^{2+}-exchanger. Downregulation of Na^+/K^+-ATPase α_2-isoform might constitute a molecular basis for altered contractile properties in CHF.

8. ERRATA

Paper 2

Paragraph 3.2., page 112, lines 8-10: The sentence should read: "...was compared to the mean fluorescence intensity of 30 distinct regions of the t-tubules."

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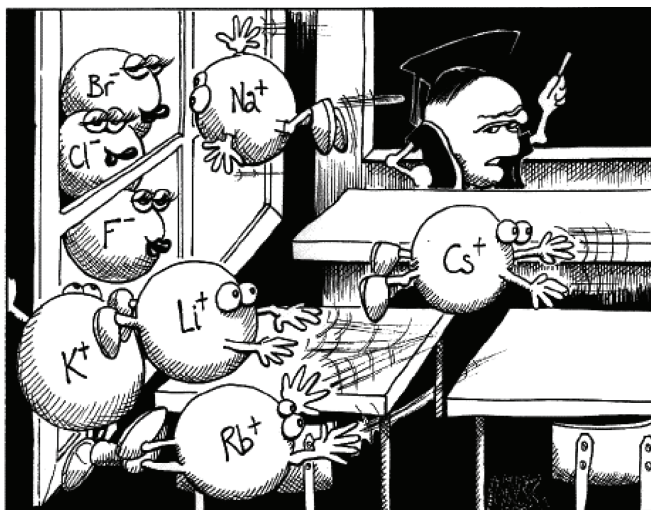
"Perhaps one of you gentlemen would mind telling me just what it is outside the window that you find so attractive...?"