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Department of Clinical Medicine

Hypothermia-induced myocardial calcium overload

A study of intracellular Ca^{2+} handling during profound hypothermia and rewarming

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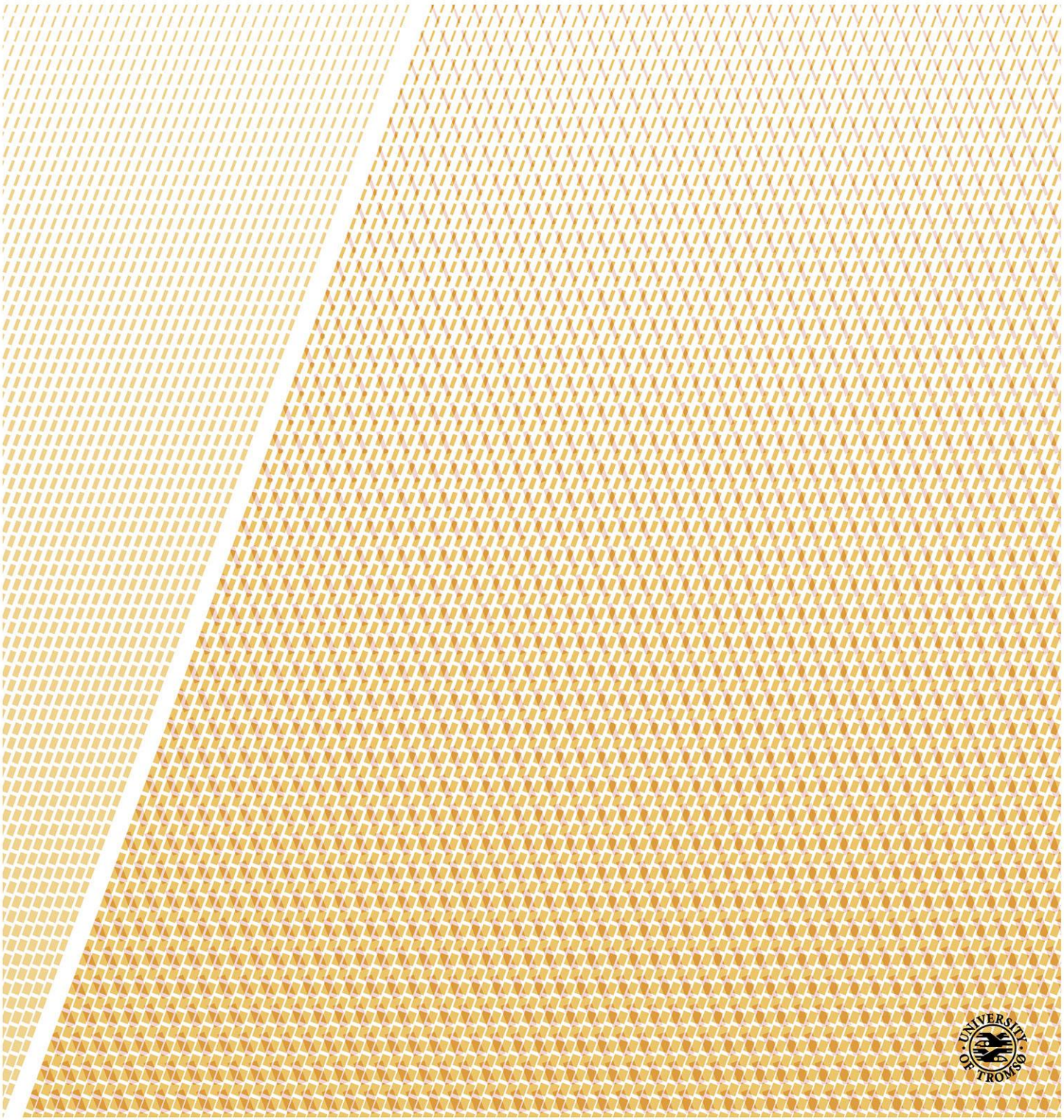
A dissertation for the degree of Philosophiae Doctor – January 2016



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1. Acknowledgements

This thesis was carried out at the Department of Clinical Medicine in the Anesthesia and Critical Care Research Group at the Arctic University of Tromsø (former University of Tromsø) during my years as a medical student while I was also doing my Master of Science in Clinical Research (research student program), between 2002 and 2008. In 2005/2006 I spent my "research year" at the Department of Physiology & Biomedical Engineering at the Mayo Clinic. From 2010 to 2016 I have finished my thesis as a PhD student at the Arctic University of Norway, partly full time and the last years part time.

I wish to express my gratitude to my supervisor Professor Torkjel Tveita who has been my guide and door-opener through all these years. His support, enthusiasm and positivity have been of great importance to my work. I also wish to thank my co-supervisor Gary C. Sieck who invited me to visit and do research in his lab at the Mayo clinic and for his helpful advices and clever thoughts.

Special thanks go to my colleague Timofei Kondratiev for his invaluable help and support during experiments at the Arctic University of Tromsø. I also wish to thank Young Soo Han and Y. S. Prakash at the Mayo Clinic for their help during experiments and in the process of developing a new model.

I also want to thank my husband, Fredrik, for his support, understanding and priorities through these years. And my daughters, Sofie and Ada, for cheering me up and reminding me of what is important.

The studies were supported by grants from the Norwegian Research Council for Science and Humanities, the University of Tromsø and the Mayo Clinic foundation.

2. Abstract

We performed randomized controlled experimental studies in an intact animal model and in isolated cardiac myocytes with the following aims:

Paper I

To establish an *in vivo* model for measuring cardiac intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) in an anesthetized rat with maintained spontaneous circulation. In this model we could sort out if alterations in Ca^{2+} homeostasis take place in response to hypothermia and rewarming.

Paper II

To investigate myocardial [Ca^{2+}]$_i$ during graded hypothermic exposure-time and after rewarming during maintained spontaneous circulation.

Paper III

To describe the Ca^{2+} homeostasis in subcellular compartments; if mitochondria buffer excessive cytosolic Ca^{2+} in single beating cardiac myocytes during hypothermia and rewarming.

Main results and conclusions

We established an *in vivo* model for measuring cardiac cell [Ca^{2+}]$_i$ which revealed that cardiac Ca^{2+} overload evolves during long-lasting profound hypothermia, and that the increase in [Ca^{2+}]$_i$ remains unchanged during rewarming. *In vitro* mitochondria buffer cytoplasmic [Ca^{2+}] ([Ca^{2+}]$_{cyt}$) during hypothermia (0,5 hs). We conclude that prolonged deep hypothermia alters cardiac myocyte Ca^{2+} handling which may deteriorate mitochondrial function contributing to the post-hypothermic myocardial failure.

3. List of papers

I. Timofei V. Kondratiev, Ragnhild M. Wold, Ellen Aasum and Torkjel Tveita. Effects of hypothermia and rewarming on myocardial calcium control in an intact animal model
Cryobiology. 2008 Feb;56(1):15-21. doi:10.1016/j.cryobiol.2007.09.005

II. Ragnhild M. Wold, Timofei V. Kondratiev and Torkjel Tveita. Effects of hypothermic exposure-time and rewarming on myocardial calcium control in an intact animal model
Acta Physiol (Oxf). 2013 Mar;207(3):460-9. doi: 10.1111/apha.12003

III. Ragnhild M. Wold, Torkjel Tveita, Niccole Schaible, Gary C. Sieck. Altered cardiac mitochondrial calcium regulation during hypothermia and rewarming
Manuscript

The papers are referred to in the text by their roman numerals.

4. List of abbreviations

AM-ester - acetoxymethyl ester

ATP - adenosine triphosphate

BSA - bovine serum albumin

$[Ca^{2+}]_{cyt}$ - cytoplasmic Ca^{2+} concentration

$[Ca^{2+}]_{mit}$ - mitochondrial Ca^{2+} concentration

$[Ca^{2+}]_i$ - intracellular Ca^{2+} concentration

CLSM - confocal laser scanning microscopy

CO - cardiac output

CPM - counts per minute

CPO - cardiac power output

dP/dt_{max} - maximum rate of rise in LV pressure

dP/dt_{min} - minimum rate of rise in LV pressure

HR - heart rate

i.v. - intravenous

K_d - dissociation constant

LV - left ventricular/ventricle

LVSP - left ventricular systolic pressure

MAP - mean arterial pressure

MCU - mitochondrial Ca^{2+} uniporter

MPTP - mitochondrial permeability transition pore

NADH - nicotinamide adenine dinucleotide

NCX - Na^+ -Ca^{2+} exchanger

PMCA - plasma membrane Ca^{2+} ATPase pump

ROS - reactive oxygen species

RyR - ryanodine receptor

SE - standard error

SR - sarcoplasmic reticulum

SV - stroke volume

TPR - total peripheral resistance

T-tubules - transverse tubules

5. Introduction

5.1 Rationale for the thesis

In Norway, the death rate from accidental hypothermia is estimated to 0,5 deaths per 100.000 inhabitants per year [1]. Today the poor and elderly, the homeless, people working outside, especially in harsh or arctic climate, are at risk [2]. These patients often present with impaired cardiac function, cardiac arrhythmias, circulatory shock or even circulatory arrest [3], and during resuscitation the mortality rate is ranging between 29% and 80% [4-6]. Thus, the consequences of accidental hypothermia may be fatal which makes this a typical low-frequency but high-impact diagnosis [4].

Hypothermia is defined as core temperature below 35ºC [7], and different under-classifications exist. The European Resuscitation Council uses the following nomenclature: mild hypothermia (32ºC - 35ºC), moderate hypothermia (28ºC - 32ºC) and severe/deep hypothermia (< 28ºC) [8]. The American Heart Association defines mild hypothermia as temperatures above 34°C, moderate between 34-30°C and severe below 30°C based on suggestions by Polderman and Herold [9].

Due to the different severities, variety in aetiology, low incidence, different rewarming methods and a relatively sparsely elucidated pathophysiology, few evidence-based treatment protocols of accidental hypothermia exist. Hence, the topic of accidental hypothermia should be studied to establish a better fundamental physiological understanding on which treatment protocols can be based. To achieve this, one needs to investigate both hypothermia-induced physiological changes as well as the consequences of rewarming.

Myocardial Ca^{2+} homeostasis is known to be affected by hypothermia. In the heart, Ca^{2+} plays a key role in the excitation-contraction coupling and is also involved in regulating adenosine triphosphate (ATP) production. In other words, fluctuations of $[Ca^{2+}]_i$ are very important for the main function of cardiac myocytes, namely; contraction and relaxation, which is often altered in victims of accidental hypothermia. Accordingly, we wanted to elucidate the impact of severe accidental hypothermia and rewarming on the regulation of myocardial $[Ca^{2+}]_i$. This information might also have relevance for mild or moderate accidental hypothermia, as well as for therapeutic hypothermia (mild hypothermia).

5.2 Hypothermia-induced cardiac contractile dysfunction

5.2.1 Categorization of the contractile dysfunction

Research indicates that during deep hypothermia (3.8 - 16ºC), there is a reduction in cardiac contractility [10-15]. By increasing exposure time to stable hypothermia, the deficit in post-hypothermic cardiac contractile function is aggravated [16, 17]. Specifically, from experimental studies in rats, we know that during cooling, there is a decrease of left ventricular (LV) end systolic volume and a subsequent increase in stroke volume (SV). However, during stable hypothermia a gradual increase in LV end systolic volume is followed by a reduction in SV [12, 16]. During the rewarming phase, isovolumetric pressure is depressed and ventricular wall shortening is reduced, whereas diastolic function is unaffected [16]. Hence, hypothermia-induced cardiac dysfunction is mainly due to compromised systolic function. A similar hypothermia-induced depression of systolic function and a reduced SV is also demonstrated in hypothermic dogs and pigs [14, 15, 18, 19].

In its fulminant form the cardiac contractile dysfunction is recognized as a progressive reduction of cardiac output (CO) and a sudden fall in arterial blood pressure [12, 17, 20, 21], and thus this is suggested to be a part of the underlying pathophysiology for the impaired cardiac function, cardiac arrhythmias, circulatory shock or even circulatory arrest presented in hypothermic/rewarmed patients [3].

5.2.2 Mechanisms underlying the contractile dysfunction

The basic pathophysiology of hypothermia/rewarming-induced contractile failure of the heart is poorly investigated. So far, researchers point out increased reactive oxygen species (ROS) formation [22, 23], impaired high energy phosphate homeostasis [17] and decreased myofilament Ca^{2+} sensitivity, resulting from increased cTnI phosphorylation [24] as possible mechanisms. In addition, results from our group suggest that rewarming following profound hypothermia is associated with a myocardial cellular stress response that involves activation of nuclear factor κB signalling and induction of autophagy [25]. At last, the hypothermia-induced contractile failure may be caused by an excessive increase of $[Ca^{2+}]_i$ [26]. This is explained by the fact that in the hypothermic heart, inhibition of ion transport mechanisms, which are involved in Ca^{2+} regulation, takes place [27-30]. Since Ca^{2+} is important for contraction and relaxation of the myocyte, such a dysregulation of Ca^{2+} homeostasis should have consequences for the function of the cardiac myocyte. This will be discussed later.

5.3 Hypothermia and myocardial $[Ca^{2+}]_i$

Hypothermia-induced increase of $[Ca^{2+}]_i$ has been reported from *in vitro* experiments using isolated cardiac myocytes [31-33] and isolated hearts [13, 28, 34, 35]. In isolated hearts, increase in $[Ca^{2+}]_i$ does not seem to normalize during rewarming [35]. While most studies have been performed in rat hearts, the impact of hypothermia/rewarming on calcium concentrations has also been examined in other species with qualitatively similar results. For example, in guinea pig hearts where hypothermia (10ºC) was maintained for 40 min, $[Ca^{2+}]_i$ was reported to be elevated, even more than in rats [36]. Compared with rat, the $[Ca^{2+}]_i$ handling of guinea pig heart is believed to more closely resemble that of the human heart, and hence, this makes it easier to draw lines into human medicine. To the best of our knowledge, no data concerning changes in myocardial $[Ca^{2+}]_i$ in in-situ hearts subjected to a non-arrested hypothermic state, has been reported to date.

5.3.1 Mechanism underlying hypothermia-induced elevation of $[Ca^{2+}]_i$

The mechanism for hypothermia-induced increase in $[Ca^{2+}]_i$ in cardiac myocytes is not fully understood but is likely multi-factorial [13]. In cardiomyocytes Ca^{2+} is best known for its central role in excitation contraction coupling. Depolarization along the sarcolemma allows Ca^{2+} in the T-tubules to enter the cell via L-type Ca^{2+} channels and induce Ca^{2+} release (43 - 55%) from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR) in the dyadic cleft (figure 1). Cooling is known to slow down SR function, and an increase in the open probability of RyR channels which may contribute to elevated $[Ca^{2+}]_i$. However, this is probably not the primary mediator of the hypothermia-induced increase of $[Ca^{2+}]_i$ [29].

$[Ca^{2+}]_{cyt}$ is extruded across the sarcolemma by the Na^+-Ca^{2+} exchanger (NCX) or the plasma membrane Ca^{2+} ATPase (PMCA) pump. $[Ca^{2+}]_{cyt}$ is also removed by SR reuptake via the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) pump or mitochondrial Ca^{2+} uptake primarily via the mitochondrial Ca^{2+} uniporter (MCU) (figure 1). The temperature sensitivity of these ion transporters may vary, and thus, an imbalance between ion pump activity and ion leaks may cause prolonged elevation of $[Ca^{2+}]_{cyt}$ ($[Ca^{2+}]_{cyt}$ loading) [37, 38]. It is widely believed that cooling increases $[Ca^{2+}]_{cyt}$ by slowing NCX, PMCA and SERCA activities. In addition, hypothermia-induced slowing of the Na^+/K^+-ATPase will increase intracellular Na^+ concentration and thus activate a reverse mode of the NCX to extrude Na^+ in exchange for Ca^{2+} influx [39].

5.3.2 $[Ca^{2+}]_i$ and inotropy

Due to Ca^{2+} influx, cardiac myofilaments are activated by the total free $[Ca^{2+}]_{cyt}$, which binds to troponin C to activate the thin filament. The degree of contractile activation depends on amount of $[Ca^{2+}]_{cyt}$ and the Ca^{2+} sensitivity of the thin filament [40]. From experiments performed at 37ºC and in room temperature, it is well known that an increase in $[Ca^{2+}]_{cyt}$ induces positive inotropy, because it increases thin filament activation and increases Ca^{2+} sensitivity [13, 29, 41-45]. However, an excessive Ca^{2+} accumulation will induce negative inotropy in the heart, and this is termed Ca^{2+} overload [46], and is expressed as impaired contractility and relaxation, hypercontracture and arrhythmias.

Initially, cooling increases contractile response (positive inotropy) recognized as an elevation of SV in intact rats (20ºC) [16], increase in developed force in rat and rabbit ventricular papillary muscle (25ºC) [29] and increased fractional shortening in pig ventricular myocytes (22ºC) [32]. This may be because of a hypothermia-induced increase in $[Ca^{2+}]_{cyt}$ [45], but an alternative explanation might be the negative staircase effect of the rat heart [16]. Deeper hypothermia decreases inotropy [16], and this is regarded as physiological event as the metabolic demands decreases with temperature [47]. However, as stated above, in severe hypothermic rats cardiac contractile dysfunction is evolving with time and persists with rewarming [15, 16]. Such negative inotropic effect is also observed in human atrial myocytes during moderate hypothermia as decreased twitch tension (rapid cooling, 31ºC) [48], in hearts from intact dogs (as reduced LV dP/dt_{max}, CO and SV at 25ºC) [14], and in Langedorff perfused (10ºC) rat and guinea pig hearts (increased LVEDP) [36]. Hypothermia-induced Ca^{2+} overload is suggested to be one of the mechanisms underlying these observations [36, 48]. However, simultaneous measurements of intracellular $[Ca^{2+}]$ and cardiac contractility during hypothermia and rewarming *in vivo* have not been performed.

The different inotropic responses to hypothermia fit the paradoxical effects of increased $[Ca^{2+}]_{cyt}$, and support our hypothesis that an increase of $[Ca^{2+}]_{cyt}$ (Ca^{2+} overload) underlies the contractile dysfunction. Moreover, studies indicate that severity of hypothermia and exposure-time are decisive factors for the inotropic response. In addition, different responses between species substantiate the fact that Ca^{2+} handling and response to cooling is species dependent.

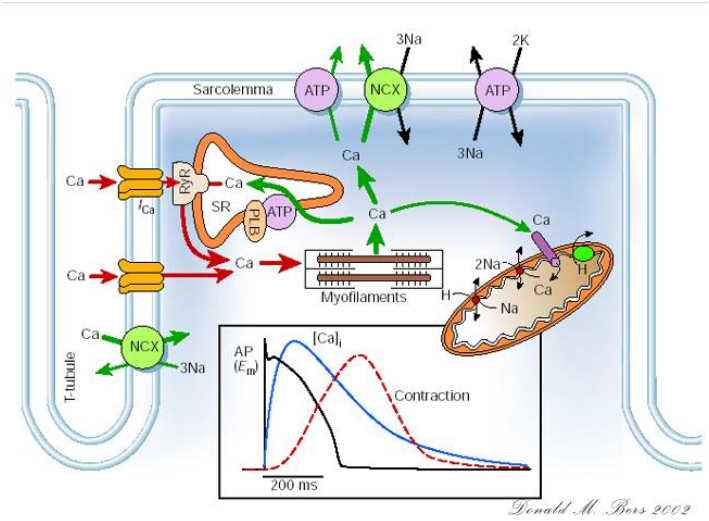

Figure 1: *The excitation-contraction coupling and cycling of Ca^{2+}. From Bers 2002 [39].*

5.4 The role of mitochondria

In mitochondria, Ca^{2+} takes part in regulating intra-mitochondrial oxidative metabolism and production of ATP (figure 2). Increased workload in the heart increases cytosolic calcium which leads to an elevated mitochondrial matrix calcium. Thus mitochondrial Ca^{2+} serves to couple the increase in work to increase in ATP production that is needed to sustain the work [49]. The mitochondrion also has an important role in Ca^{2+} buffering since it is capable of sequestering considerable amonts of Ca^{2+} by forming Ca^{2+} phospate complexes [50] (table 1). This buffering effect is observed at physiological conditions such as changed heart rate and/or changed workload and under unphysiological conditions with higher [Ca^{2+}] [51].

Details of how Ca^{2+} enters the mitochondria is not completely settled; Ca^{2+} enters the mitochondrial matrix, driven by its electrochemical gradient, through the MCU. In addition, the leucine-zipper-EF-hand-containing transmembrane protein 1 (LETM 1) may also be participating in Ca^{2+} influx [49, 51] (figure 2).

The Na^+-Ca^{2+}-Li^+ exchanger (NCLX) is the most important mechanism for extrusion of $[Ca^{2+}]_{mit}$ using the influx of sodium down its electrochemical gradient [49, 52]. In addition, to limit excessive accumulation of matrix Ca^{2+} the mitochondrial permeability transition pore (MPTP) might act as a Ca^{2+} release valve [51, 53, 54]. Opening of the MPTP is suggested to be activated by cellular stress (i.e. high $[Ca^{2+}]_i$ and $[ROS]_i$) [55]. Opening leads to increased permeability of the inner mitochondrial membrane to large molecules. Thus prolonged opening consequently results in degradation of the inner mitochondrial membrane potential and ultimately necrotic cell death [56-58].

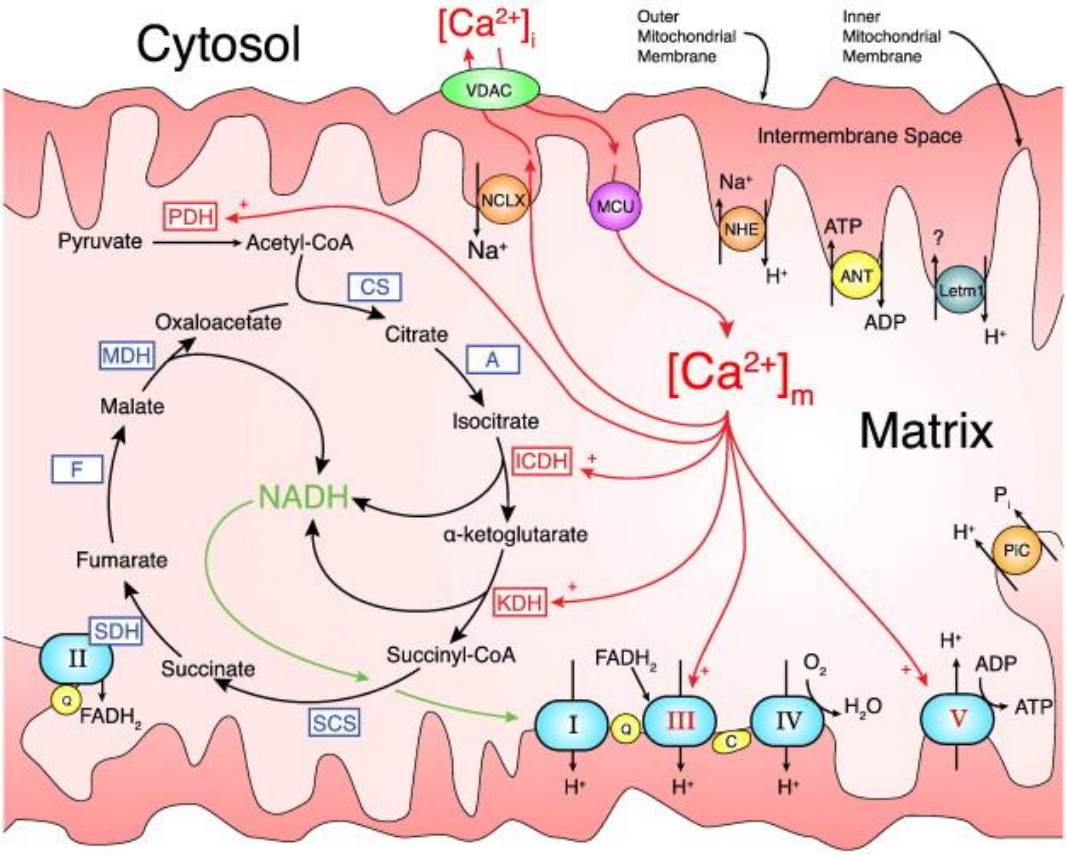

Figure 2: *Mitochondrial metabolism components. Red arrows indicate Ca^{2+} interactions/pathways, and enzymes with known Ca^{2+} sensitivities are red. The five complexes responsible for oxidative phosphorylation are light blue ovals. Pyruvate dehydrogenase (PDH); citrate synthase (CS); aconitase (A); isocitrate dehydrogenase (ICD); a-ketoglutarate dehydrogenase (KDH); succinyl CoA synthetase (SCS); succinate dehydrogenase (SDH); fumarase (F); malate dehydrogenase (MDH); mitochondrial Ca^{2+} uniporter (MCU); mitochondrial Na^{+}/Ca^{2+} exchanger (NCLX); Leucine zipper-EF-hand containing transmembrane protein 1 (Letm1); cytochrome C (C); ubiquinone (Q); voltage-dependent anion channel (VDAC); ATP/ADP translocase (ANT); inorganic phosphate carrier (PiC); and the mitochondrial Na^{+}/H^{+} exchanger (NHE). From Williams et al. 2015.*

Localization	**[Ca^{2+}] (µM = µmol/L of cytosol)**
Extracellular	1000 µM (free)
Cytosol	0,15/1,00 µM (diastole/systole) (free)
Dyadic cleft	10 -100 µM (free)
Ca^{2+} hot spots/microdomains	50 - 100 µM (free)
Sarcoplasmic reticulum	100 µM (total)
Mitochondria	100 - 10 000 µM (total)

Table 1: *Values of [Ca^{2+}] (µM = µmol/L of cytosol) in a cardiac myocyte [40, 59].*

5.4.1 Observations in the hypothermic/rewarmed mitochondria

Although there is considerable information regarding basal mitochondrial function at room temperature (equivalent to severe hypothermia) and protective effects of therapeutic hypothermia, few have focused on the actual effects of cooling in this organelle. Riess et al. have reported that hypothermia induces an increase of $[Ca^{2+}]_{mit}$ in cold perfused isolated hearts (17ºC, 50 min) which reverses with rewarming [60]. During hypothermia (13-15ºC) and rewarming *in vivo*, and during hypothermia *in vitro*, decreased ATP production, increased ROS generation and damaged mitochondrial ultrastructure have been reported [16, 22, 60]. These observations encouraged us to have a closer look at the Ca^{2+} dynamics between the mitochondria and cytosol.

5.4.2 Possible consequences of altered mitochondrial Ca^{2+} homeostasis

According to observations by Riess et al., and the fact that $[Ca^{2+}]_{cyt}$ *in vitro* rises with cooling, it is likely that mitochondria buffer the excessive $[Ca^{2+}]_{cyt}$, and at some point $[Ca^{2+}]_{mit}$ overload may occur. $[Ca^{2+}]_{mit}$ is a critical regulator of mitochondrial function [61] and mitochondrial calcium overload is a primary trigger of necrotic cell death [62]. Suggested mechanisms behind mitochondrial dysfunction and cell death are mitochondrial depolarization, uncoupling of oxidative phosphorylation, decreased ATP synthesis, ROS generation and mitochondrial swelling [40, 56-58, 63-70]. If these events take place during hypothermia/rewarming, the contractile function of the cardiac myocyte will be affected. We hypothesized that hypothermia/rewarming-induced myocardial contractile failure may arise from mitochondrial Ca^{2+} buffering of excessive $[Ca^{2+}]_{cyt}$ which results in perturbation of mitochondrial function through the same mechanisms as listed above. To the best of our

knowledge, simultaneous measurements of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ in single cardiac myocytes during hypothermia and rewarming has not been previously performed.

6. Aims of the thesis

The central goal of the present thesis was to shed light on the aspects of myocardial Ca^{2+} handling at low temperatures which may be crucial to cardiac function and force development during hypothermia and rewarming. To achieve this, we intended to measure cardiac $[Ca^{2+}]_i$ in intact animals with spontaneous circulation, and measure $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ in electrically stimulated single cardiac myocytes during hypothermia and rewarming.

The specific aims of the present study were as follow:

6.1 Paper I

To establish an *in vivo* model for measuring cardiac $[Ca^{2+}]_i$ in an anesthetized rat with maintained spontaneous circulation. In this model we could sort out if alteration in Ca^{2+} homeostasis takes place in response to hypothermia and rewarming

6.2 Paper II

To elucidate changes in myocardial $[Ca^{2+}]_i$ during graded hypothermic exposure-time during maintained spontaneous circulation.

6.3 Paper III

To investigate the Ca^{2+} homeostasis in subcellular compartments; if mitochondria buffer excessive $[Ca^{2+}]_{cyt}$ in single beating cardiac myocytes during hypothermia and rewarming.

7. Methods and methodological considerations

7.1 Choice of animal species

The rat was chosen as experimental animal in all studies. *In vivo* studies have shown that this animal maintains spontaneous electromechanical cardiac activity down to about 13ºC, and it is less susceptible to arrhythmias compared to larger animals during hypothermic exposure [71]. The *in vivo* rat model is well established and have successfully been used on our laboratory for hypothermic research for years [16, 16, 47, 72-74, 74, 75, 75] making it a reliable and reproducible experimental animal model. This anesthetized, intact animal model of accidental hypothermia needs minimal surgical intervention, and at the same time it offers the possibility to sample a wide range of hemodynamic, as well as laboratory, variables.

The advantage obtained when using isolated cells, lies in the possibility to isolate the aspects of cooling and rewarming to the cardiac myocyte alone. There are no hormonal or neuronal influences or coronary flow that needs to be considered. However, in isolated cells the possible regulations that occur between myocytes, endothelium, intercellular space, cardiac lymph and plasma are not present. This makes the isolated cell model a comparative reductionist model that cannot mimic all aspects of hypothermia. Many experiments in cardiac myocytes are conducted at room temperature (deep hypothermia). In one aspect, this makes it easier to compare our results from hypothermia with other studies on rat cardiac myocytes. On the other hand, to compare results with studies describing basic cellular functions at room temperature without normothermic "controls", might bias our conclusions.

Extrapolation of data obtained at the single cellular level, or isolated hearts, to the intact heart should be done with caution. In addition, previous studies suggest species-dependent differences in SR function and cellular responses to hypothermia [29, 76]. Again, one should be careful making conclusions for human from rat experimental models.

7.2 In vivo experiments (paper I and II)

7.2.1 Instrumentation and measurements of hemodynamic variables

All experimental protocols were approved by Norwegian Research Authority and conducted on anesthetized male Wistar rats according to European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18.III.1986).

Anesthesia was introduced intraperitoneally by 55 mg/kg body weight pentobarbital sodium and fentanyl 50 μg/kg body weight, followed by a continuous infusion of 7,5

mg/kg/hour pentobarbital sodium and 50 μg/kg/hour fentanyl through an intravenous (i.v.) line in the right jugular vein extended to the right auricle. In the hypothermic groups the infusion was terminated when cooling was started due to hypothermia created anesthesia and reduced drug metabolism, and reinstituted during rewarming at 34 - 37ºC. The normothermic group was given anaesthesia during the whole experiment. The animals were monitored by toe-pinch for any signs of discomfort. This is a well-established method for testing the effects of analgesic drugs in rodents and has been extensively tested in rats[77].

Both pentobarbital and fentanyl potentially affect the cardiovascular system by lowering blood pressure and inducing bradycardia. In addition, they may depress respiration. The doses used were standardized, and from our experience with this model, these side-effects are not a problem. However, in addition to monitoring blood pressure, we also analysed blood gasses.

The rat was placed on the operating table in a supine position. The trachea was opened and a tracheal tube inserted. All animals had spontaneous and sufficient ventilation at core temperatures above 20ºC. At core temperatures below 20ºC normoventilation was achieved by a volume-controlled small animal respirator using room air.

Mean arterial pressure (MAP) was continuously monitored through a fluid filled catheter introduced into the left femoral artery. LV pressure was measured through a fluid-filled catheter (20 G) placed in the left ventricle via the right common carotid artery. LV pressure was differentiated to obtain the maximum rate of rise in LV pressure (LV dP/dt_{max}). Catheters were connected to transducers, and the signals from the pressure transducer were amplified to 0 - 10 V and passed to a 12-bit analogue to digital converter. Signal processing and analysis were performed with the help of a special computer program developed at our department using a commercially available software package. Right atrium was catheterized through the right external jugular vein and used for injection for cardiac output (CO) measurements (thermodilution technique) and anesthesia infusion. A thin thermocouple, connected to a thermocouple controller was positioned in the aortic arch through the right femoral artery to monitor core temperature and temperature changes during CO measurements. Skin wounds were thereafter sutured and heat-exchanging tubes were placed in oesophagus and lower bowels.

7.2.2 Core cooling and rewarming

The animals in the hypothermic groups were cooled and rewarmed by circulating cold or warm water through U-shaped polyethylene tubes placed in oesophagus and lower bowels. In

addition, the double-layered operating table made of hollow aluminium was circulated by temperature-adjusted water. Core temperature was continuously monitored using the thermocouple wire positioned in the aortic arch via right femoral artery, connected to the thermocouple controller. Cooling lasted about 60 min, while rewarming lasted about 120 min.

7.2.3 Cardiac output measurements

CO was measured by thermodilution technique [78] which is reported to a be a reliable method also at low temperatures [79, 80]. One must take in to account that CO measured by thermodilution technique in small animals, especially at low flow, could be overestimated [79, 81, 82] due to the greater heat diffusion in these animals. However, by maintaining a constant room temperature and comparing CO at same core temperatures during cooling and rewarming in the same animal, we have eliminated the influence of this error.

We injected saline (0,1 – 0,15 ml) pre-cooled in ice water through the i.v. line positioned in the right auricle. Change in temperature was recorded from the thermocouple positioned in the aortic arch through the right femoral artery. Thermodilution curves were recorded on a Linearcorder. These curves were digitalized with a digitizing table, and CO was calculated with a program designed with the LabView package.

$$\mathbf{CO = (T_{blood} - T_{inj}) \times Vol_{inj} \times 60/A \times Y_{res} \times X_{res}}$$

$\mathbf{T_{blood}}$ -blood temperature (ºC),
$\mathbf{T_{inj}}$ -temperature of the injected saline (ºC),
$\mathbf{Vol_{inj}}$ -volume of the injected saline (ml),
A -area under the curve (mm^2),
$\mathbf{Y_{res}}$ -resolution of y-axis (ºC/mm) and
$\mathbf{X_{res}}$ -resolution of x-axis (sec/mm).

CO was calculated as mean of three consecutive measurements.

7.2.4 Blood gases measurements

Blood gases, oxygen saturation, pH and [Ca^{2+}] were measured in 0,15 ml arterial blood samples taken from femoral artery after surgery (37ºC), at 15ºC and after rewarming to 37ºC in the hypothermic group and three times in normothermic control group (at baseline and after

3 and 5 hs at 37ºC). Samples were analysed by a blood gas analyser. In accordance to the advice of Ashwood et al., the results were not temperature corrected (pH-stat), meaning we used the alpha-stat method [83]. The blood gasses data are presented in paper II only.

7.2.5 Measurements of $[Ca^{2+}]_i$ in hearts

To measure $[Ca^{2+}]_i$ the technique of using radiolabelled Ca^{2+} ($^{45}Ca^{2+}$) [84] was adapted to *in vivo* use. This method cannot distinguish between intracellular pools of Ca^{2+}, and consequently only total $[Ca^{2+}]_i$ was measured. Calculations of $[Ca^{2+}]_i$ are based on measured concentrations of Ca^{2+} in plasma and concentrations of $^{45}Ca^{2+}$ in plasma and in heart tissue (specific radioactivity). For use in intact animals some adjustments in the technique were necessary. Specifically, in an intact animal model, in contrast to isolated hearts where isotopes in the perfusate equilibrates in heart tissue within less than 1 min [84], a significant time interval is necessary after intravascular injection to equilibrate the distribution of $^{45}Ca^{2+}$ throughout the body. In pilot experiments, $^{45}Ca^{2+}$ was measured in blood samples collected from several minutes to several hours after injection of $^{45}Ca^{2+}$. During the first minute after injection, a rapid decline in $^{45}Ca^{2+}$ activity was observed. Stable levels were achieved before 2 hours after time of injection, and 2 hours were considered adequate to achieve equilibrium of $^{45}Ca^{2+}$ distribution in the tissues. During the experimental protocol 20 μCi of $^{45}Ca^{2+}$, diluted in 0,5 ml saline, was injected via the catheter introduced to the left-ventricle catheter after instrumentation was completed.

Before termination of experiments, arterial blood samples were drawn to obtain specific activity and concentration of $^{45}Ca^{2+}$ and total Ca^{2+}. At the end of experiment, hearts were exteriorized through a sternotomy and then immediately perfused in a Langendorff system over 3 min with Krebs-Henseleit bicarbonate buffer at room temperature to wash out extracellular $^{45}Ca^{2+}$.

In pilot experiments hearts were perfused by this procedure for 15 min and samples of the coronary effluent containing $^{45}Ca^{2+}$ were collected following few seconds to several minutes to obtain $^{45}Ca^{2+}$ activity from the extracellular space. We found that already after 1 min, $^{45}Ca^{2+}$ activity in the coronary perfusate decreased to a level that remained unchanged during the next 4 to 14 min. This corresponds with data from Tani and Neely who described $t_{1/2}$ for $^{45}Ca^{2+}$ washout to be about 4 seconds [85]. From these experiments, a 3 min washout period was decided to be used.

The total myocardial $[Ca^{2+}]_i$ was expressed as µmol/g dry tissue and found by assuming that specific radioactivity $^{45}Ca^{2+}$-total Ca^{2+} ratio for heart and plasma were the same ($[SpR]_{plasma}/[Ca^{2+}]_{plasma} = [RR]_{heart}/[Ca^{2+}]_i$). In order to determine the specific activity of the isotope in plasma, an arterial blood sample (1 ml), drawn immediately before terminating the experiment, was centrifuged for 5 min at 10000 rpm, and specific radioactivity in plasma ($[SpR]_{plasma}$) was measured by using a liquid scintillation spectrometer in 10 ml of scintillation fluid with addition of 0,1 ml plasma. Plasma Ca^{2+} concentrations ($[Ca^{2+}]_{plasma}$) in the same samples were determined using the blood gas analyser.

After washout of extracellular $^{45}Ca^{2+}$, hearts were quickly freeze-clamped with metal clamps precooled in liquid nitrogen, and the hearts were dried and homogenated. Thereafter, 80-90 mg of tissue homogenate was extracted in 1 ml ice-cold 0.42 M perchloric acid for 10 min and then centrifuged for 10 min at 3000 rpm. The residual intracellular $^{45}Ca^{2+}$ was counted by a liquid scintillation spectrometer in 5 ml of scintillation fluid with addition of 0,2 ml of homogenate extract. The $[Ca^{2+}]_i$ was calculated as follows:

$$\mathbf{[Ca^{2+}]_i = [RR]_{heart} \times [Ca^{2+}]_{plasma} / HW \times [SpR]_{plasma}}$$

$[RR]_{heart}$ - residual specific radioactivity in heart tissue after washout of extracellular $^{45}Ca^{2+}$, [CPM/ml]
$[Ca^{2+}]_{plasma}$ - plasma concentration of Ca^{2+}, [µmol/ml]
HW - weight of heart tissue homogenate extracted in 1 ml of perchloric acid, [g/ml]
$[SpR]_{plasma}$ - the specific plasma radioactivity, [CPM/ml]

7.3 In vitro experiments (Paper III)

7.3.1 Dissociation of cardiac myocytes

Adult male Sprague-Dawley rats were injected intramuscularly with ketamine (60 mg/kg) and xylazine (2,5 mg/kg) for anesthesia. Hearts were exteriorized through a sternotomy, and cardiac myocytes were enzymatically dissociated using a Langendorff perfusion-based technique with oxygenated Tyrodes solution containing 1,2 mM $CaCl_2$, followed by a Ca^{2+} free Tyrode solution with 10,0 mM creatinine and 20,0 mM taurine. Cardiac myocytes are susceptible to the enzymatic digestion process, and hence to avoid Ca^{2+} overload, a Ca^{2+} free buffer was used. After 5 min, 0,2 mM $CaCl_2$, 0,6 mg/ml Type II Collagenase and 0,1 mg/ml

protease with 1% bovine serum albumin (BSA) were added to stabilize the enzymes and increase protein solubility.

The Ca^{2+} concentration was progressively increased in graded steps to 1,2 mM. After the heart tissue was sufficiently digested by the enzymes, the LV was excised, scissor-minced and gently triturated to obtain single myocytes in fresh Tyrodes solution containing 2% BSA.

7.3.2 Loading with fluorescent Ca^{2+} indicators

Enzymatically-dissociated cardiac myocytes were loaded simultaneously with 5 μM rhod-2/AM and 5 μM fluo-3/AM in 1,2 mM Ca^{2+} Tyrodes solution for 45 min at room temperature in a dark room. Loading dyes at room temperature is a compromise between the fact that processing of AM-esters by esterases increases with temperature. However, by increasing temperature some cell types actively endocytose material from the incubation medium in to organelles by vesicular traffic [86]. Fluo-3 is mainly loaded to the cytosol.

Following incubation, cells were washed and placed in a two-coverslip based closed perfusion chamber equipped with stimulating electrodes. Ca^{2+} tolerance and cell viability were evaluated by the absence of plasma membrane blebs and spontaneous $[Ca^{2+}]_{cyt}$ waves and oscillations (with only infrequent spontaneous contractions). Cells that displayed spontaneous contractions, and/or did not maintain an elongated morphology, were excluded from further analysis.

Loading with AM ester, up to 80 % of the intracellular rhod-2, a hydrophobic organic cation with a delocalized positively charged structure, loads over the mitochondrial membrane into the mitochondrial lumen which are both negatively loaded. In mitochondria, cleavage of AM ester groups by esterases liberates the Ca^{2+} sensitive form of rhod-2 (bearing multiple nondelocalized negative charges) which is not membrane permeant and thus trapped in the mitochondrial lumen. This way, compartmentalization of rhod-2 can be used to monitor intramitochondrial Ca^{2+} signals. However, we have to take into account that the membrane permeant AM-esters never loads to only one compartment [86]. In a small set of experiments, the intracellular localization of rhod-2 was compared to MitoTracker Green to verify mitochondrial loading of rhod-2. Confocal microscopy of cells co-loaded with MitoTracker Green and rhod-2 was achieved using a laser scanning confocal microscope system with argon (488 nm) and solid state (561 nm) lasers. Images were captured and then pseudo-colored and analyzed. The pattern of rhod-2 fluorescence was predominantly within mitochondria compared to cytosolic areas with no MitoTracker Green fluorescence (figure 1,

paper III). In a second set of experiments, the distinction between $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ was also confirmed by exposing the cardiac myocytes to a mitochondrial uncoupler, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which prevents$[Ca^{2+}]_{mit}$ accumulation. Exposure of cardiac myocytes to CCCP resulted in reduced rhod-2 fluorescence (indicating that these signals were primarily of mitochondrial origin), but increased fluo-3 fluorescence (reflecting $[Ca^{2+}]_{cyt}$ elevation).

7.3.3 Imaging with confocal laser scanning microscopy

To measure cytosolic and mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$), we imaged fluo-3 and rhod-2 fluorescence using confocal laser scanning microscopy (CLSM). Fluorescence microscopy (epi-fluorescence) differs from most traditional microscopic techniques in that the visible light in the microscope eyepieces is not the original light emitted by the light source. The light you see is actually fluorescence emitted at specific wavelengths from a fluorophore (e.g., fluo-3 emitted at 525 nm, and rhod-2 emitted at 581 nm) within the cardiac myocytes after activation by a specific laser wavelengths (e.g., fluo-3 excited at 488 nm and rhod-2 excited at 552 nm) [87].

Confocal, as in CLSM, means that the illuminating and fluorescent lightpaths have the same focus. This can be achieved by different methods, but commonly a pinhole aperture is used to limit divergent light, and a dichroic mirror produces a sharp, intense point of illumination at a specific depth within the myocytes. The optical section thickness is determined by the combination of the pinhole dimension and the numerical aperture of the objective lens. A second dichroic mirror processes the fluorescent light from the specimen (myocytes) and allows only light information from the illuminated point to reach the detectors [88] (fig. 3). This way, confocal microscopy produces images in which out-of-focus fluorescence is eliminated. Electrical stimulation and superfusion does not interfere with confocal imaging [88].

The source of the illumination light is a laser. This light is an intense monochromatic light with low divergence, a property that optimizes the effectiveness of the confocal light path. Monochromaticity of the light source eliminates chromatic aberration/refraction of wavelengths. In addition, the high-intensity illumination increases the speed of image acquisition [87].

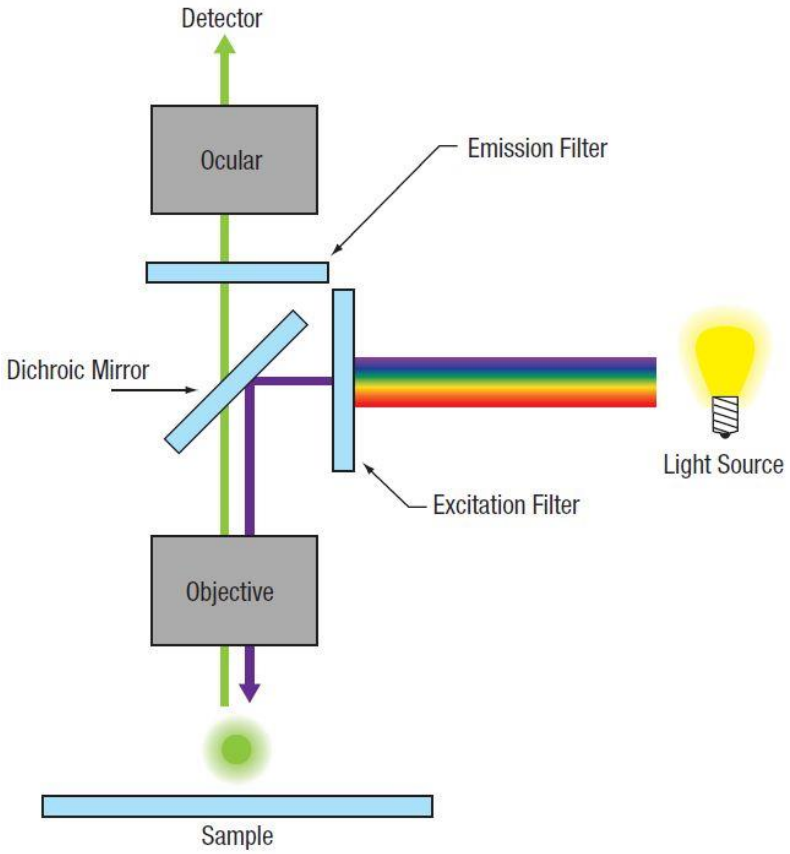

Figure 3. *Principles of confocal laser scanning microscopy (CLSM). From www.thorlabs.de [89].*

In general, fluorescent Ca^{2+} indicators can be excited at wavelengths ranging from UV to infrared, including the wavelengths encompassing visible light. The advantages of visible-wavelength Ca^{2+} indicators are less cytotoxicity and their emissions are in regions of the electromagnetic spectrum where cellular autofluorescence and background scattering are less severe. Rhod-2 and Fluo-3 are excited by visible light and have excitation-emission wavelengths that do not interfere which make them suitable to monitor cytosolic and mitochondrial [Ca^{2+}] simultaneously [86]. Fluo-3 is one of the most suitable visible-wavelength Ca^{2+} indicators for CLSM with a K_d for Ca^{2+} estimated to be 390 nM which corresponds with physiologic $[Ca^{2+}]_{cyt}$ levels [90]. Fluo-3 enhances its fluorescence 40 fold upon binding Ca^{2+} [88]. Rhod-2 has a K_d for Ca^{2+} at 570 nM, and its fluorescence enhances on binding Ca^{2+} by 100 fold [90]. The K_d of rhod-2 and fluo-3 are quite low, hence these probes may be saturated during Ca^{2+} overload [91].

Fluo-3 and rhod-2 are sensitive to photobleaching [92]. However, problems associated with excessive illumination were obviated in the present study by the use of the VisiTech Eye real-time confocal system, which employs an optical scanning system that has a very short dwell time for laser illumination. Furthermore, cells were illuminated only during limited data recording periods in order to minimize phototoxicity and photobleaching.

Fluo-3 and rhod-2 are high affinity Ca^{2+} indicators, and hence they also work as Ca^{2+} buffers [92]. In other words, since dyes must bind Ca^{2+} in order to report its concentration, they can perturb the true level of $[Ca^{2+}]_i$. This may also change the intracellular environment [93]; for example, the regulation of $[Ca^{2+}]_i$ are controlled by the free $[Ca^{2+}]$. Especially, during rapid changes of $[Ca^{2+}]$, the buffering effect of a present Ca^{2+} indicator becomes more apparent [94]. In the present work temperature changes might induce such a rapid change. To avoid excessive buffering of $[Ca^{2+}]_i$ we limited the concentrations of fluo-3AM and rhod-2AM during loading.

In our experiment normal Tyrodes solutions containing 137 mM NaCl, 5,4 mM KCl, 0,5 mM $MgCl_2$, 1,2 mM CaCl, 0,33 mM NaH_2PO_4, 10,0 mM HEPES, 10,0 mM glucose (pH adjusted to pH 7,38 at 27ºC) was perfused at 1 ml/min. The $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses of 2 - 3 myocytes per coverslip were obtained using individual, software-defined regions of interest (20*20 µm). Cells were visualized at 400X (40X/1,3 oil-immersion lens; 640 x 480 pixels per frame; 30 frames s^{-1}, optical section thickness of 1 µm). Since the experimental protocols were fairly lengthy, cells were excited only during data recording.

7.3.4 Measurements of fluorescence

Fluo-3 and rhod-2 are non-ratiometric dyes and hence fluorescence intensities cannot be exactly calibrated to provide quantitiative estimates of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$. This also make $[Ca^{2+}]$ measurements susceptible to errors stemming from changes in dye concentration and motion artifacts [91].

Calibration experiments were performed in order to interpret the measurement of fluorescent indicators fluo-3 and rhod-2 during temperature changes. This was accomplished by exposing the salt form of each dye (rhod-2 tripotassium salt and fluo-3 pentapotassium salt) to a range of free $[Ca^{2+}]$ solutions at three temperatures (17, 27 and 37ºC), two pH levels (7.2 and 7.4) and a constant ionic strength (150 mM). The fluorescent levels associated with each $[Ca^{2+}]$ was measured using a benchtop fluorometric plate reader. The dissociation constant (K_d) was calculated for each Ca^{2+} curve by finding the x-intercept of the double log

plot of free [Ca^{2+}] versus fluorescence ratio. The fluorescent intensity of rhod-2 and fluo-3 increased during hypothermia due to a decrease in K_d during transition from 37ºC to 17ºC. The K_d of Rhod-2 decreased by 6,7% at 27ºC and 42% at 15ºC while the K_d of fluo-3 decreased by 14,4% at 27ºC and 35% at 15ºC. Therefore, fluorescence measurements during hypothermia were scaled accordingly by the proportional change in K_d (relative to 37ºC) at each temperature.

7.3.5 Temperature control

Accurate temperature control of the cell suspension was achieved by heating/cooling the water jacket surrounding the cell chamber, as well as heating/cooling the perfusion tubing close to where it entered the cell chamber, using a thermostat controlled perfusion water bath. Temperature was continuously monitored with a thermocouple wire, placed right next to the water jacket surrounding the cell chamber and connected to a digital thermometer. In pilot experiments, temperature stability and consistency of changes were verified.

7.3.5 Electrical stimulation

Myocytes were electrically stimulated via a stimulator attached to the platinum wires within the cell chamber. Twitch stimulation was performed at a constant rate of 0,5 Hz, using single pulses of 5 ms duration and voltages ranging from 4 - 7 V. This stimulation rate is equivalent to 30 bpm in normal tissue and was chosen because the spontaneous pacing rate in isolated hearts at 15ºC is about 30 bpm [95].

7.4 Experimental protocols

7.4.1 Paper I, $[Ca^{2+}]_i$ after hypothermia and rewarming

Animals were divided into two groups; one hypothermic group (n = 7) cooled to 15ºC, maintained at 15ºC for 4 hs and then rewarmed to normothermia, and one normothermic control group (n = 7), kept for 5 hs at 37ºC, which served as control. $^{45}Ca^{2+}$ was given two hours before baseline measurement.

Hemodynamic measurements: In the hypothermic group, hemodynamic variables were measured at 37ºC, 30ºC and 20ºC during cooling and rewarming, and every hour during stable hypothermia. In the control group, measurements were sampled every hour.

Blood gas measurements: In the hypothermic group blood gases were measured after instrumentation, after cooling to 15ºC and after rewarming to 37ºC. In normothermic controls blood gases were measured at baseline and after 3 and 5 hs.

$[Ca^{2+}]_i$ measurements: Before termination of the experiment, a blood sample was taken to measure $[Ca^{2+}]_{plasma}$ and $[^{45}Ca^{2+}]_{plasma}$. The experiment was terminated by excising the heart which immediately was prepared for measurements of $[^{45}Ca^{2+}]_i$.

7.4.2 $[Ca^{2+}]_i$ during hypothermia and after rewarming, paper II

Animals were divided into four groups – one hypothermic group kept at 15ºC for 30 min, a second hypothermic group kept at 15ºC for 4 hs, a rewarmed group following 4 hs at 15ºC and a time matched control group kept at 37ºC for 5 hs. $^{45}Ca^{2+}$ was given two hours before baseline measurement.

Hemodynamic measurements, blood gas measurements and $[Ca^{2+}]_i$ measurements were the same as in paper I.

7.4.3 Paper III, mitochondrial and cytosolic Ca^{2+} signals during hypothermia and rewarming

Cell stability was verified over a 15 min period of electrical stimulation (room temperature). All experiments started at a temperature of 37ºC. The temperature was changed in the following steps as $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ transients were recorded: 37ºC (regarded as control temperature for reference), 27ºC (towards cooling), 15ºC (hypothermia temperature; maintained for 30 min), 27ºC (towards rewarming) and 37ºC (rewarmed temperature). In order to achieve exact temperature of cells at each step, cells were allowed to stabilize for a 2-

3 min before recording $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ transients. Total time of one experiment was about 180 min.

For each recording, baseline $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ were measured. Transient $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses evoked by electrical field stimulation were analyzed from a series of 10 twitch responses per set. From the 10 twitch responses, average peak of the evoked $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ were determined as well as the average amplitude (peak minus baseline) of these responses (figure 2, paper III). The average time to the peak of the evoked $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses was also measured as well as the average time to 50% decay of evoked $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses (figure 2, paper III).

7.5 Statistics

Differences between *in vivo* groups (paper I and II) were compared using two-sample independent Student's *t*-test. Hemodynamic changes within one group were assessed in the cooled and rewarmed group by One-Way ANOVA.

Data from individual cardiac myocytes (paper III) were averaged and statistical comparisons were performed across different time points during hypothermia and rewarming using a One-Way ANOVA. 10 cardiac myocytes, obtained from at least 5 animals, were analyzed.

In all experiments results are presented as mean ± SE, and a p-value < 0.05 was considered significant.

8. Summary of results

8.1 Paper I

In this paper we compared changes in hemodynamic function during profound long lasting hypothermia, 4 hs 15ºC, and total intracellular Ca^{2+} ($[Ca^{2+}]_i$) after rewarming to time-matched normothermic controls.

8.1.1 Myocardial $[Ca^{2+}]_i$

Myocardial $[Ca^{2+}]_i$ from the hypothermic/rewarmed rats was 3,01 ± 0,43 µmol/g dry weight which is almost a seven fold elevation compared to normothermic controls were myocardial $[Ca^{2+}]_i$ was 0,44 ± 0,05 µmol/g dry weight (figure 3, paper I). These data are comparable to values obtained in normothermic *in vitro* experiments [36].

8.1.2 Hemodynamics

With respect to hemodynamics, stability of the model was confirmed as no alterations were measured during 5 hs of experiments in the normothermic control group. During cooling there was a reduction of most hemodynamic variables, as shown in numerous previous experiments during similar conditions [15, 16, 96]. After 4 hs at 15ºC, mean arterial pressure (MAP) and left ventricular systolic pressure (LVSP) were reduced by 65 - 70%, whereas heart rate (HR) and maximum rate of rise in left ventricular pressure (LV dP/dt_{max}) were reduced to 13 and 4 % respectively, compared to their pre-hypothermic baseline values. Due to technical limitations, we were not able to measure cardiac output (CO) below 20ºC. At 20ºC, CO was reduced by 38%. Total peripheral resistance (TPR) was increased by almost 30%, while stroke volume (SV) was increased almost three-fold at 20ºC (figure 1 and 2, Paper I).

Hemodynamic function following rewarming was compared to pre-hypothermic values; after rewarming CO and SV were both significantly reduced to 77% of baseline values, while TPR was increased by 28%. LVSP and LV dP/dtmax were reduced to 91% and 83%, respectively, but did not reach statistical significance. HR and MAP, returned to baseline levels, and LV end-diastolic pressure (LVEDP) remained unchanged during the experiment.

8.2 Paper II

In this paper we studied changes in $[Ca^{2+}]_i$ and hemodynamic function during profound hypothermia for 0,5 and 4 hs at 15ºC, and after rewarming.

8.2.1 Myocardial $[Ca^{2+}]_i$

In the group maintained at 15ºC for 0,5 h $[Ca^{2+}]_i$ was 0,55 ± 0,10 μmol/g dry weight which was not statistically different from normothermic control levels, 0,35 ± 0,06 μmol/g dry weight, after 5 hs. The hypothermic value is five to six times below values from studies done in isolated hearts exposed 10ºC for 40 min [28, 35, 36] (table 2). In the group maintained at 15ºC for 4 hs, $[Ca^{2+}]_i$ was 2,33 ± 0,53 μmol/g dry weight, which is more than a six fold increase compared to the normothermic control group. In the post-hypothermic group, following 4 hs at 15ºC, $[Ca^{2+}]_i$ was 1,97 ± 0,38 μmol/g dry weight, which is almost a six fold increase from control. The latter result corresponds with to data from paper I as well as other experiments [36].

8.2.2 Hemodynamics

Stability of the model, with respect to hemodynamics, was confirmed as no alterations were measured during 5 hs of experiments in the normothermic control group.

Cooling to 20ºC reduced CO and cardiac power output (CPO) by 37% and 70% respectively. TPR was decreased by 34%, whereas SV was almost three-fold increased. Subsequent cooling to 15ºC continued to reduce MAP and LVSP by 75 - 80%. HR and LV dP/dt_{max} were 14% and 6% respectively of their normothermic values. At 15ºC 0.5 h, hemodynamic parameters were unchanged from corresponding values at 15ºC 0 min. At 15ºC 4 hs, MAP, LVSP, LV dP/dtmax, LV dP/dt_{min} were all significantly lowered, 21 - 50% compared with 15ºC 0h.

Following 4 hs at 15ºC and then rewarmed to 20ºC, CO, COP and SV were all significantly reduced compared their corresponding values during cooling. When rewarming was completed, CO, COP and SV were all significantly reduced from baseline values (start of experiment). TPR was increased by 60% from baseline values. HR and MAP returned to pre-hypothermic levels. LVEDP remained unchanged during the experiments. The contractility index dP/dt_{max} remained lower than pre-hypothermic and cooling values during the rewarming process, but this was not significant, neither were changes in LVSP.

8.3 Paper III

In our third paper we investigated the intracellular distribution of Ca^{2+} between cytosol and mitochondria during hypothermia and rewarming in single, electrically stimulated cardiac myocytes.

The cardiac myocytes appeared elongated without plasma membrane blebs. They responded well to field stimulation, and spontaneous contractions were rarely observed. Transient elevations in $[Ca^{2+}]_{mit}$ consistently followed evoked $[Ca^{2+}]_{cyt}$ responses across all temperatures and throughout the experiments.

8.3.1 Calibration Results

In a set of experiments using the Fabiato program we found that K_d decreased with temperature and presumed change in pH for both fluo-3 and rhod-2. Therefore, fluorescence measurements were scaled according to the known change in K_d with temperature and presumed change in pH for each indicator.

8.3.2 Baseline $[Ca^{2+}]_{mit}$ and $[Ca^{2+}]_{cyt}$

Compared to control values at 37ºC pre-hypothermia, baseline $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ were significantly decreased at 15ºC (figure 4, paper III). After 30 min at 15ºC and with subsequent rewarming, $[Ca^{2+}]_{cyt}$ tended to increase and baseline $[Ca^{2+}]_{mit}$ returned progressively to control (pre-hypothermia) levels.

8.3.3 Evoked $[Ca^{2+}]_{mit}$ and $[Ca^{2+}]_{cyt}$ responses

Electrical stimulation evoked transient elevations of $[Ca^{2+}]_{cyt}$ in cardiac myocytes that were matched by elevations of $[Ca^{2+}]_{mit}$. The peak of the evoked $[Ca^{2+}]_{cyt}$ responses decreased during hypothermia and returned to pre-hypothermic levels during rewarming (figure 5, paper III). The amplitude (peak minus baseline) decreased during hypothermia and rewarming as baseline tended to increase. In contrast, both the peak and amplitude of the evoked $[Ca^{2+}]_{mit}$ responses were unchanged during hypothermia at 15ºC and rewarming.

During hypothermia and after rewarming, electrical stimulation often evoked secondary $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses. These secondary $[Ca^{2+}]$ responses were present in more than 50 % of cardiac myocytes.

8.3.4 Time to peak $[Ca^{2+}]_{mit}$ and $[Ca^{2+}]_{cyt}$

The time to peak of both $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses were markedly increased (~150 - 230 %) during hypothermia at 15ºC compared to pre-hypothermic levels (figure 6a, paper III). With rewarming, the time to peak of both $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses returned to control (pre-hypothermia) levels.

8.3.5 Time to 50% decay $[Ca^{2+}]_{mit}$ and $[Ca^{2+}]_{cyt}$

The time to 50% decay of both evoked $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses were markedly increased (~200 - 300%) during hypothermia at 15ºC compared to control pre-hypothermia levels (figure 6b, paper III). With rewarming the time to 50% decay of both evoked $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses returned to pre-hypothermic values.

As a result of the prolongation of the evoked $[Ca^{2+}]$ responses, the integrated $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ responses were markedly elevated during hypothermia at 15ºC compared to control pre-hypothermia levels ($P<0.05$). This prolongation reflected a hypothermia-related $[Ca^{2+}]$ overload in cardiac myocytes.

9. General discussion

In the presented thesis we developed a method for measuring total cardiac $[Ca^{2+}]_i$ with $^{45}Ca^{2+}$ technique. The results demonstrate that hypothermia-induced elevation of myocardial $[Ca^{2+}]_i$ takes place in an intact animal, and the increase is not reversed by rewarming. In the *in vitro* study, we found that during hypothermia mitochondrial buffer a possible excessive $[Ca^{2+}]_{cyt}$.

9.1 Hypothermia-induced cytosolic Ca^{2+} loading

The mechanism for cytosolic Ca^{2+} loading during hypothermia is not known and is likely multi-factorial. In cardiac myocytes the components regulating intracellular Ca^{2+} homeostasis are affected by temperature; the prevailing theory to explain hypothermia-induced elevation of $[Ca^{2+}]_i$ is that the Na^+/K^+-ATPase is markedly inhibited by cooling [27], Q_{10} coefficient 3,0 [28], which leads to an elevation of $[Na^+]_i$. The rise in $[Na^+]_i$ may entail a reversed mode of the Na^+-Ca^{2+} exchanger (NCX), Q_{10} coefficient 1,35 [13, 29], resulting in elevated Ca^{2+} uptake [39]. The fact that the NCX is less affected by temperature than the Na^+/K^+ ATPase, makes this possibility likely.

Hypothermia also increases the action potential duration [29, 97] and reduces the rate of inactivation of the Ca^{2+} current [13, 97, 98]. Moreover, hypothermia inhibits sarco-/endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2) [30], and the rate of SR Ca^{2+} release per unit Ca^{2+} current is increased [32]. Among the Ca^{2+} extrusion mechanisms, sarcolemmal Ca^{2+} ATPase and the MCU are slowed by cooling [39]. These mechanisms may all elevate cytoplasmic Ca^{2+} at low temperatures.

9.2 Consequences of prolonged hypothermia

In paper II we found that cardiac $[Ca^{2+}]_i$ after long-term hypothermia *in vivo* (15ºC, 4 hs) was substantially elevated from short-term hypothermia (15ºC, 0,5 h) (which was not significantly increased from normothermic control). This indicates that the time-aspect of exposure to hypothermia is crucial to $[Ca^{2+}]_i$, and that hypothermia *in vivo* does not induce an immediate rise in myocardial $[Ca^{2+}]_i$. This time-dependent elevation of $[Ca^{2+}]_i$ might be reflected by the observed reduction in contractile function -LVSP and LV dP/dt_{max} were all significantly lowered (21 - 50%) after 4 hs at 15ºC compared to 15ºC 0 min (paper II). The latter observations are supported by others [16, 17].

Conversely, it is well known that cooling *in vitro* rapidly elevates $[Ca^{2+}]_i$ and $[Ca^{2+}]_{cyt}$ [13, 28, 29, 31, 32, 34, 35, 38, 99-104], and the results from paper III might also suggest that

hypothermia induces elevation of Ca^{2+} transients in single cardiac myocytes. The divergent findings *in vivo* and *in vitro* may be explained by the possibility that *in vivo* hearts better tolerate low temperature since *in vitro* hearts, or cells, are exposed to a dramatic change of their environment. However, according to table 2, the *in vitro* experiments show a smaller increase in $[Ca^{2+}]_{cyt}$ when compared to the converted values from the *in vivo* hearts. Hence, the $^{45}Ca^{2+}$ technique used in the *in vivo* experiments might not be sensitive enough to detect smaller changes of $[Ca^{2+}]_i$ already evolving during the first minutes of hypothermia.

Contractile failure induced by elevated $[Ca^{2+}]_i$ is defined as "Ca^{2+} overload" [46]. Based on our *in vivo*-results, we assume that calcium overload takes place during long-lasting hypothermia. Ca^{2+} overload is extensively studied following myocardial ischemia/reperfusion where Ca^{2+} overload is regarded an important substrate for reduced contractile function. The underlying mechanisms are not clear, but the following are suggested to be involved are SR Ca^{2+} release to cytosol giving rise to $[Ca^{2+}]_{cyt}$ oscillations/delayed after-depolarizations that may induce arrhythmias and myofibrillar hypercontracture [68, 105], reduced membrane potential and variation in action potential duration [106], $[Ca^{2+}]_{mit}$ overload, dysfunction and damage (discussed in detail below) involving increase in ROS [22, 23] and impaired high energy phosphate homeostasis [17], and decreased myofilament Ca^{2+} sensitivity resulting from increased cTnI phosphorylation [24]. In addition, deep hypothermia itself prolongs SR Ca^{2+} release and reuptake over the cardiac cycle. This results in diastolic Ca^{2+} loading of the cytosol which impairs relaxation and contractility will subsequently decrease [13].

Author **Year**	**Method**	**$[Ca^{2+}]_{pre}$**	**$[Ca^{2+}]_{hypo}$**	**$[Ca^{2+}]_{rew}$**	**Comment**
Wang 1999	Indo-1 Rat cardiac myocytes	**0,14**	**0,22**		Not contracting 30ºC vs. 10ºC **$[Ca]_i$ [µmol/L cytosol]**
Shutt 2008	Fura-2 Guinea pig cardiac myocytes	**0,06/0,1**	**0,1/0,26**		Contracting (diastolic/systolic) 37ºC vs. 22ºC **$[Ca]_i$ [µmol/L cytosol]**
Stowe 1999	Indo-1 Rat Langendorff perfused heart	**0,19/1,2**	**0,65/2,20**		Paced, 220 bpm (diastolic/systolic) 37ºC vs. 17ºC **$[Ca^{2+}]_{cyt}$ [µmol/L cytosol]**
Aasum 1997	$^{45}Ca^{2+}$ Rat Langendorff perfused heart	1,0 **0,49***	3,83 **1,88***	1,8 **0,88***	37ºC vs. 15ºC(40min) $[Ca^{2+}]_i$ [µmol (g dry wt)$^{-1}$] **$[Ca^{2+}]_{cyt}$ [µmol/L cytosol]***
Aasum 1997	$^{45}Ca^{2+}$ Guinea pig perfused heart	0,7 **0,34***	9,3 **4,56***	2,1 **1,03***	37ºC vs. 15ºC(40min) $[Ca^{2+}]_i$ [µmol (g dry wt)$^{-1}$] **$[Ca^{2+}]_{cyt}$ [µmol/L cytosol]***
Kondratiev 2008	$^{45}Ca^{2+}$ Heart from intact rat	0,44 **0,22***		3,01 **1,47***	37ºC vs. 37ºC (rewarmed from 4hs 15ºC) $[Ca^{2+}]_i$ [µmol (g dry wt)$^{-1}$] **$[Ca^{2+}]_{cyt}$ [µmol/L cytosol]***
Paper II 2013	$^{45}Ca^{2+}$ Heart from intact rat	0,35 **0,17***	0,55-2,33 **0,27-1,14***	1,97 **0,97***	37ºC vs. 15ºC (0,5 and 4 hs) $[Ca^{2+}]_i$ [µmol (g dry wt)$^{-1}$] **$[Ca^{2+}]_{cyt}$ [µmol/L cytosol]***

Table 2: *$[Ca]_i$ (µmol/L cytosol (**bold**)) or (µmol (g dry wt)$^{-1}$) and $[Ca^{2+}]_{cyt}$ (µmol/L cytosol) before, during and after hypothermia in vitro and in vivo. * means conversion to $[Ca^{2+}]_{cyt}$, according to Bers [40]. However, this conversion might not correct for increased mitochondrial Ca^{2+} buffering.*

9.3 Mitochondrial Ca^{2+} buffering

The mitochondrion is capable of storing enormous amounts of Ca^{2+} (up to 10,000 µM, table 1) bound to inorganic phosphate [50]. This way the free $[Ca^{2+}]_{mit}$ is kept in the low µM range allowing a precise regulation of ATP production [50]. Due to this Ca^{2+} buffering, mitochondrial Ca^{2+} handling likely protects the cell against Ca^{2+} overload [50, 107-112].

According to table 2, hypothermia increases $[Ca^{2+}]_i$ by a factor of 4 and 7 during cooling [34, 113], but in isolated cardiac myocytes $[Ca^{2+}]_{cyt}$ is only increased by 0,2 – 3,4 fold (17ºC, exposure-time unknown) [13]. This might reflect that the primary hypothermia-induced increase of $[Ca^{2+}]_{cyt}$ is buffered, and this is supported by the results from paper III.

In this paper, the observed elevation of transient $[Ca^{2+}]_{mit}$ responses (time to peak and 50% decay) may reflect that the mitochondria buffer hypothermia-induced excessive $[Ca^{2+}]_{cyt}$. Conversely,we found that baseline $[Ca^{2+}]_{mit}$ is decreased during hypothermia which might support the fact that the MCU is markedly inhibited at lower temperature [39]. Hence, mitochondrial Ca^{2+} uptake should be decreased with cooling. Moreover, since time to 50%

decay of $[Ca^{2+}]_{mit}$ was increased during hypothermia, it is likely that mitochondrial Ca^{2+} extrusion through the NCLX is slowed, and this will, if anything, contribute to the elevation of $[Ca^{2+}]_{mit}$.

Whether the observed mitochondrial Ca^{2+} buffering has consequences for mitochondrial function, with possible relation to hypothermia-induced contractile dysfunction, is not elucidated. In general, mitochondrial Ca^{2+} uptake activates mitochondrial ROS production [55, 68]. Ca^{2+} overload or increased ROS production may cause MPTP opening which leads to mitochondrial inner membrane permeabilization, membrane potential dissipation, impaired respiratory chain function, halt of mitochondrial ATP synthesis, organelle swelling, and outer membrane rupture [56, 58]. Ultimately the outcome will be loss of plasma membrane integrity and cell rupture characterized as necrotic cell death [57]. MPTP activation may also induce autophagy and cellular stress responses which is known to take place during rewarming from hypothermia [25]. Excessive activation of autophagy can also trigger autophagic cell death [109].

Hypothermia/rewarming *in vivo* is known to decrease ATP synthesis and damage mitochondrial ultra-structure [15, 16]. Cold perfusion (17ºC, 50 min) in isolated hearts attenuates electron transport and causes formation of ROS [22, 60]. These observations suggest that the mechanisms listed above also take place during hypothermia and rewarming due to mitochondrial Ca^{2+} buffering of excessive $[Ca^{2+}]_{cyt}$.

9.4 Outcome of rewarming

With respect to contractile function of the heart and the outcome from accidental hypothermia, the calcium homeostasis after rewarming is an important factor. In paper I and II, hypothermia-induced elevated $[Ca^{2+}]_i$ persisted during rewarming and disclosed in concert with cardiac contractile dysfunction. The latter corresponds with earlier results from this model [16] and is supported by others [11, 12]. Contractile dysfunction during deep hypothermia and after rewarming is also reported as reduced maximal Ca^{2+}-activated force and decreased twitch force in papillary muscle (15ºC) [24] and as reduced maximal Ca^{2+}-activated LVP and impaired relaxation in isolated hearts (17ºC) [13]. Contractile failure induced by elevated $[Ca^{2+}]_i$ is defined as Ca^{2+} overload [46]. Based on our results, we assume that Ca^{2+} overload takes place during and after rewarming.

In paper III, the changes of time to peak and time to 50% decay of $[Ca^{2+}]_{cyt}$ were normalized during rewarming. Baseline, however, first decreased during cooling but tended to increase during rewarming (figure 4, paper III). In cold perfused isolated hearts (10 – 15ºC, 40

min) a hypothermia-induced Ca^{2+} accumulation was only partially normalized following rewarming [28, 34, 35]. Transient responses of $[Ca^{2+}]_{mit}$ in paper III were unaltered during the experiment (figure 5, paper III). Other researchers have reported that hypothermia-induced elevation of $[Ca^{2+}]_{mit}$, attenuated electron transport and moderate formation of ROS were reversed by rewarming and did not lead to functional impairment in cold perfused (17ºC, 50 min) isolated hearts [22, 60].

The observed $[Ca^{2+}]_{mit}$ decrease of time to peak and 50% decay during rewarming might indicate that mitochondria is releasing Ca^{2+} during rewarming. This may be caused by a NCLX release of Ca^{2+} as a consequence of restored function of pumps and exchangers, and normalized $[Ca^{2+}]_{cyt}$.

The decrease in baseline of $[Ca^{2+}]_{mit}$ during hypothermia and the unchanged amplitude does not support a possible increase of $[Ca^{2+}]_{mit}$. The *in vitro* hypothermia-protocols are fairly short-lasting with respect to in a clinical setting of accidental hypothermia. This leads us to speculate if our results in paper III, which partly indicates elevation $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$, were only a modest increase. *In vivo*, the hypothermia-induced increase of $[Ca^{2+}]_i$ was detected after 4 hs and persisted during rewarming. After 0,5 h at 15ºC, no elevation of $[Ca^{2+}]_i$ was detected. This might suggest that reducing time of cold exposure is crucial for preserving Ca^{2+} homeostasis and possibly for optimizing cardiac function during and after rewarming.

In paper III we did not measure myocyte contractility, so we cannot absolutely relate the observed changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ to myocyte function. However, during hypothermia and rewarming we found decreased amplitude of $[Ca^{2+}]_{cyt}$, possibly symbolizing a reduction of evoked SR Ca^{2+} release. In more than 50% of our cells, spontaneous release of Ca^{2+} was observed as fluorescent waves and after-contractions during and after rewarming. This phenomenon is explained by the accepted mechanism of delayed after-depolarizations. The SR refills with Ca^{2+} from the previous beat and the RyR recovers from refractoriness, and the high luminal SR $[Ca^{2+}]$ causes local SR Ca^{2+} release events (Ca^{2+} sparks) [105]. Some cells also ceased to contract and underwent changes in morphology. These observations make it tempting to suggest that the impaired Ca^{2+} handling entails post-hypothermic contractile dysfunction also *in vitro*.

9.5 Future directions

Simultaneous recordings of cytosolic and mitochondrial Ca^{2+} signals are challenging. One major problem is that although rhod-2 accumulates predominantly in the mitochondria, some may remain in the cytosol and thus be affected non-specifically by $[Ca^{2+}]_{cyt}$ changes [86]. In

separate studies where rhod-2 was loaded into cells together with MitoTracker Green, it was shown that rhod-2 fluorescence is predominantly co-localized with MitoTracker Green fluorescence. Other potential problems include the risk of cross-talk between rhod-2 and fluo-3 given the marginal spectral overlap as well as dye leakage from the cells over the more than two hours course of our experiments. In future studies, we will explore cytosolic [Ca^{2+}] and mitochondrial [Ca^{2+}] separately as well as ratiometric alternatives to cytosolic [Ca^{2+}] measurement such as the combination of fluo-4 and fura red. Another option is to define regions of interest within mitochondria (using mitotracker) and outside according to Delmotte et al. [114].

10. Final conclusions

The main objective of the present thesis was to investigate Ca^{2+} handling in cardiac tissue during profound hypothermia and rewarming. By measuring total Ca^{2+} content and Ca^{2+}distribution between cytosol and mitochondria, we have elucidated the Ca^{2+} homeostasis during hypothermia and rewarming. The results might point out mechanisms behind the hypothermia-induced contractile dysfunction.

The main conclusions, based on results from the experiments are:

- Rewarming following 4 hs of profound hypothermia induces an almost seven-fold increase in intracellular myocardial tissue calcium content and a significant reduction in cardiac output and stroke volume. This indicates that hypothermia/rewarming-induced calcium overload in the myocardium may contribute to the post-hypothermic myocardial failure.

- In the intact, beating heart *in vivo,* cardiac Ca^{2+} overload evolves during long-lasting profound hypothermia, and the increase in $[Ca^{2+}]_i$ remains unchanged during rewarming. These results indicate that the observed post-hypothermic Ca^{2+} overload *in vivo* is established during hypothermia, that duration of hypothermia is crucial to $[Ca^{2+}]_i$ and that the myocardial Ca^{2+} overload is not reversed by rewarming. This adds to our understanding of the pathophysiology of hypothermia rewarming-induced contractile dysfunction.

- During hypothermia, mitochondria buffer excessive $[Ca^{2+}]_{cyt}$. The results partly indicates that hypothermia induces elevation of $[Ca^{2+}]_{mit}$ which is released to cytosol during rewarming. Hypothermia-induced accumulation of $Ca^{2+}{}_{mit}$ may prevent a cytosolic Ca^{2+} overload but possibly also deteriorates mitochondrial function and energy production. These novel data elucidates the consistency between Ca^{2+} overload and mitochondrial function. However, the cause and effect relationship between hypothermia-induced changes in $[Ca^{2+}]_{mit}$ and hypothermia-induced mitochondrial dysfunction and contractile dysfunction still needs to be elucidated.

11.Reference list

Reference List

[1] Filseth OM. Aspects of experimental cooling and rewarming with special reference to accidental hypothermia; incidence of death from accidental hypothermia. 15-16. 2012. University of Tromsoe.
Ref Type: Thesis/Dissertation

[2] Dietrichs ES. Pharmacological approaches to management of hypothermia-induced cardiac dysfunction. 2015.
Ref Type: Thesis/Dissertation

[3] MacLean, Emslie-Smith. Accidental hypothermia. 1977.

[4] Ploeg GJ, Goslings JC, Walpoth BH, Bierens JJ. Accidental hypothermia: rewarming treatments, complications and outcomes from one university medical centre. Resuscitation 2010;81:1550-5.

[5] Silfvast T, Pettila V. Outcome from severe accidental hypothermia in Southern Finland—a 10-year review. Resuscitation 2003;59:285-90.

[6] Vassal T, Benoit-Gonin B, Carrat F, Guidet B, Maury E, Offenstadt G. Severe accidental hypothermia treated in an ICU: prognosis and outcome. Chest 2001;120:1998-2003.

[7] Reuler JB. Hypothermia: pathophysiology, clinical settings and management. Ann Intern Med 1978;89:519-27.

[8] Soar J, Perkins GD, Abbas G, Alfonzo A, Barelli A, Bierens JJ et al. European Resuscitation Council Guidelines for Resuscitation 2010 Section 8. Cardiac arrest in special circumstances. Resuscitation 81[10], 1400-1433. 2010.
Ref Type: Journal (Full)

[9] Polderman KH, Herold I. Therapeutic hypothermia and controlled normothermia in the intensice care unit: practical considerations, side effects, and cooling methods. Crit Care Med 2009;37:1101-20.

[10] Kondratiev TV, Myhre ESP, Simonsen Ø, Nymark TB, Tveita T. Cardiovascular effects of epinephrine during rewarming from hypothermia in an intact animal model. J Appl Physiol 2006;100:457-64.

[11] Morray JP, Pavlin E. Oxygen delivery and consumption during hypothermia and rewarming in the dog. Anesthesiology 1990;72:510.

[12] Popovic VP, Kent KM. Cardiovascular responses in prolonged hypothermia. Am J Physiol 1965;209:1069-74.

[13] Stowe DF, Fujita S, An J, Paulsen RA, Varadarajan SG, Smart SC. Modulation of myocardial function and [Ca2+] sensitivity by moderate hypothermia in guinea pig isolated hearts. Am J Physiol 1999;277:2321-32.

[14] Tveita T, Mortensen M, Hevrøy O, Refsum H, Ytrehus K. Experimental hypothermia: Effects of core cooling and rewarming on hemodynamics, coronary blood flow and myocardial metabolism in dogs. Anesth Analg 1994;79:212-8.

[15] Tveita T, Ytrehus K, Myhre ESP, Hevrøy O. Left ventricular dysfunction following rewarming from experimental hypothermia. J Appl Physiol 1998;85.

[16] Tveita T, Skandfer M, Refsum H, Ytrehus K. Experimental hypothermia and rewarming: changes in mechanical function and metabolism of rat hearts. J Appl Physiol 1996;80:291-327.

[17] Tveita T. Rewarming from hypothermia. Newer aspects on the pathophysiology of rewarming shock. International Journal of Circumpolar Health 2000;59:260-6.

[18] Filseth OM, How OJ, Kondratiev T, Gamst TM, Tveita T. Post-hypothermic cardiac left ventricular systolic dysfunction after rewarming in an intact pig model. Critical Care 2010;14:R211.

[19] Tveita T, Hevrøy O, Refsum H, Ytrehus K. Coronary endothelium-derived vasodilation during cooling and rewarming of the in situ heart. Can J Physiol Pharmacol 1994;77:56-63.

[20] Blair E, Montgomery AV, Swan H. Posthypothermic circulatory failure. I. Physiologic observations on the circulation. Circulation 1956;13:909-15.

[21] Steen PA, Milde JH, Michenfelder JD. The detrimental effects of prolonged hypothermia and rewarming in the dog. Anesthesiology 1980;52:224-30.

[22] Camara AKS, Reiss ML, Kevin LG, Novalija E, Stowe DF. Hypothermia aguments reactive oxygen species detected in the guinea pig isolated perfued hearts. Am J Physiol 2003;Heart Circ Physiol:1289-99.

[23] Reilly AM, Williams DA, Dusting GJ. Dexamethasone inhibits endotoxin-induced changes in calcium and contractility in rat isolated papillary muscle. Cell Calcium 1999;26:1-8.

[24] Han YS, Tveita T, Prakash YS, Sieck GC. Mechanisms underlying hypothermia-induced cardiac contractile dysfunction. Am J Physiol 2010;298:H890-H897.

[25] Tveita AA, Dietrichs ES, Tveita T. Myocardial gene expression profiling of rewarming shock in a rodent model of accidental hypothermia. Cryobiology 2012;64:201-10.

[26] Clanachan AS. Contribution of protons to post-ischemic Na(+) and Ca(2+) overload and left ventricular mechanical dysfunction. J Cardiovasc Electrophysiol 2006;17:141-8.

[27] Isenberg G, Trautwein W. Temperature sensitivity of outward current in cardiac purkinje fibers. Evidence of electrogenicity of active transport. Pflugers Arch 1975;358:225-34.

[28] Steigen T, Aasum E, Larsen TS. Effect of fatty acids on myocardial calcium control during hypothermic perfusion. J Thorac Cardiovasc Surg 1994;107:233-41.

[29] Shattock MJ, Bers DM. Inotropic response to hypothermia and the temperature-dependence of ryanodine action in isolated rabbit and rat ventricular muscle: implications for excitation-contraction coupling. Circ res 1987;61:761-71.

[30] Labow RS, Hendry PJ, Meek E, Keon WJ. Temperature affects human cardiac sarcoplasmic reticulum energy-mediated calcium transport. J Mol Cell Cardiol 1993;25:1161-70.

[31] Bers DM, Bridge JHB, Spizer KW. Intracellular Ca2+ transients during rapid cooling contractures in guinea-pig ventricular myocytes. J Physiol 1989;417:537-53.

[32] Shutt RH, Howlett SE. Hypothermia increase the gain of excitation-contraction coupling in guinea pig ventricular myocytes. Am J Physiol Cell Physiol 2008;295:C692-C700.

[33] Wang SQ, Zhou ZQ. Alpha-stat calibration of indo-1 fluorescence and measurement of intracellular free calcium in rat ventricular cells at different temperatures. Life Sciences 1999;65:871-7.

[34] Aasum E, Larsen TS. Pyruvate reverses fatty-acid-induced depression of ventricular function and calcium overload after hypothermia in guinea pig hearts. Cardiovasc Res 1997;33:370-7.

[35] Aasum E, Larsen TS. Stimulation of carbohydrate metabolism reduces hypothermia-induced calcium load in fatty acid-perfused rat hearts. J Mol Cell Cardiol 1997;29:527-34.

[36] Aasum E, Larsen TS. Different tolerance to hypothermia and rewarming of isolated rat and guinea pig hearts. Cryobiology 1999;38:249.

[37] Navas JA, Anderson W, Marsh JD. Hypothermia increases calcium content of hypoxic myocytes. Heart Circ Physiol 1990;28:333-9.

[38] Puglisi JL, Bassani RA, Bassani JWM, Amin JN, Bers DM. Temperature and relative contribution of Ca transport systems in cardiac myocyte relaxation. Am J Physiol 1996;270:1772-8.

[39] Bers DM. Cardiac excitation-contraction coupling. Nature 2002;415:198-205.

[40] Bers DM. Ca sources and sinks; mitochondrial Ca transport. In. Excitation-contraction coupling and cardiac contractile force. Dordrecht: Kluwer Academic Publishers; 2001; p. 56-62.

[41] Bers DM. Calcium fluxes involved in control of cardiac myocyte contraction. Circulation research 2000;87:281.

[42] Bers DM. Cardiac inotropy and calcium mismanagement. In. Excitation-contraction coupling and cardiac contractile force. Dordrecht: Kluwer Academic Publishers; 2001; p. 273-331.

[43] Kusuoka H, Ikoma Y, Futaki S, Suga H, Kitabatake A, Kamada T et al. Positive ionotropism in hypothermia partially depends on an increase in maximal Ca2+ - activated force. Heart Circ Physiol 1991;30:1005-10.

[44] Liu B, Wang IC, Belke DD. Effect of temperature and pH on cardiac myofilament Ca2+ sensitivity in rat and ground squirrel. Am J Physiol 1993;33:108.

[45] Sprung J, Stowe DF, Kampine JP, Bosnjak ZJ. Hypothermia modifies anesthetic effect on contractile force and Ca transients in cardiac purkinje fibers. Am J Physiol 1994;267:H725-H733.

[46] Vassalle M, Lin CI. Calcium overload and cardiac function. J Biomed Sci 2004;11:542-65.

[47] Kondratiev TV, Flemming K, Myhre ESP, Sovershaev MA, Tveita T. Is oxygen supply a limiting factor for survival during rewarming from profound hypothermia. Am J Physiol 2005;291:441-50.

[48] Goetzenich A, Schroth SC, Emmig U, Autschbach R, Pieske B, Rossaint R et al. Hypothermia exerts negative inotropy in human atrial preparations: in vitro-comparison to rabbit myocardium. J Cardiovasc Surg (Torino) 2009;50:239-45.

[49] Finkel T, Menazza K, Holstrom KM, Parks RJ, Liu J, Sun J. The Ins and Outs of Mitochondrial Calcium. Circ res 2015;116:1810-9.

[50] Wei A, Liu T, Winslow R. Dynamics of matrix-free Ca2+ in cardiac mitochondria: two components of Ca2+ uptake and role of phosphate buffering. The Journal of General Physiology 2012;139:465-78.

[51] Williams G, Boyman L, Lederer W. Mitochondrial calcium and the regulation of metabolism in the heart. J mol cell cardiol 2015;78:35-45.

[52] Crompton M, Kunzi M, Carafoli E. The calcium-induced and sodium-induced effluxes of calcium from heart mitochondria. Evidence for a sodium-calcium carrier. Eur J Biochem 1977;558:549-58.

[53] Elrod J, Wong R, Shikha M, Vagnozzi RJ, Sakthievel B, Goonasekera SA et al. Cyclophilin D controls mitochondrial pore-dependent Ca2+ exchange, metabolic flexibility and propensity for heart failure in mice. J Clin Invest 2010;120:3680-7.

[54] Elrod J, Molkentin J. Physiologic Functions of Cyclophilin D and the Mitochondrial Permeability Transition Pore. Circ J 2013;77:1111-22.

[55] Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev 2014;94:909-50.

[56] Duchen M. Mitochondria, calcium-dependent neuronal death and neurodegenerative disease. Pflugers Arch Eur j Physiol 2012;464:111-21.

[57] Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. Trends Biochem Sci 2007;32:37-43.

[58] Rasola A, Bernardi P. Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. Cell Calcium 2011;50:222-33.

[59] Dorn G, Maack C. SR and mitochondria: Calcium cross-talk between kissing cousins. J Mol Cell Cardiol 2013;55:42-9.

[60] Riess ML, Camara AKS, Kevin LG, An J, Stowe DF. Reduced reactive O2 species formation and preserved mithochondrial NADH and [Ca2+] levels during short-term 17C ischemia in intact hearts. Cardiovasc Res 2004;61:580-90.

[61] Boyman L, Chikando A, Williams G. Calcium movement in cardiac mitochondria. Biophys J 2014;107:1289-301.

[62] Murphy E, Steenbergen C. Preconditioning: the mitochondrial connection. Annu Rev Physiol 2007;69:51-67.

[63] Tani M. Mechanisms of Ca2+ overload of reperfused ischemic myocardium. Annu Rev Physiol 1990;52:543-59.

[64] Hüser J, Rechenmacher CE, Blatter LA. Imaging the permeability pore transition in single mitochondria. Biophys J 1998;74:2129-37.

[65] Lemasters JJ, Theruvath TP, Zhong Z, Nieminen AL. Mitochondrial calcium and the permeability transition in cell death. Biochimica et Biophysica Acta 2009;1787:1395-401.

[66] Grimm S, Brdiczka D. The permeability transition pore in cell death. Apoptosis 2007;12:841-55.

[67] Halestrap AP. What is the mitochondrial permeability transition pore. J Mol Cell Cardiol 2009;46:821-31.

[68] Dedkova EN, Blatter LA. Mitochondrial Ca2+ and the heart. Cell Calcium 2008;44:77-91.

[69] Davidson SM, Duchen MR. Effects of NO on mitochondrial function in cardiomyocytes: Pathophysiological relevance. Cardiovasc Res 2006;71:10-21.

[70] Ghafourifar, P, Richter C. Mitochondrial Nitric Oxide Synthase Regulates Mitochondrial Matrix pH. Biol Chem 1999;380:1025-928.

[71] Covino BG, D'Amato HE. Mechanism of ventricular fibrillation in hypothermia. Circ res 1962;10:148-55.

[72] Kondratiev TV, Tveita T. Effects of sympathetic stimulation during cooling on hypothermic as well as posthypothermic hemodynamic function. Can J Physiol Pharmacol 2006;84:985-91.

[73] Kondratiev TV, Myhre ESP, Simonsen T, Nymark TB, Tveita T. Cardiovascular effects of epinephrine during rewarming from hypothermia in an intact animal model. J Appl Physiol 2006;100:457-64.

[74] Tveita T, Ytrehus K, Skandfer M, Øian P, Helset E, Myhre ESP et al. Changes in blood flow distribution and capillary function after deep hypothermia in rat. Can J Physiol Pharmacol 1996;73:376-81.

[75] Tveita T, Myklebust R, Ytrehus K. Changes in myocardial ultrastructure induced by cooling as well as rewarming. Res Exp Med 1998;197:243-54.

[76] Bassani RA, Bers DM. Na-Ca exchange is required for rest-decay but not for rest-potentiation of twitches in rabbit and rat ventricular myocytes. J mol cell cardiol 1994;26:1335-47.

[77] Collier H, Warner B, Skerry R. Multiple toe-pinch method for testing analgesic drugs. Br J of Pharmacol Chemother 1961;17:28-40.

[78] Fegler G. Measurement of cardiac output in anesthetized animals by a thermo-dilution method. Q J Exp Physiol Cogn Med Sci 1954;39:153-64.

[79] Hanwell A, Linzell JL. Validation of the thermodilution technique for the estimation of cardiac output in the rat. Comp Biochem Physiol A 1972;41:547-657.

[80] Merrick SH, Hessel EA 2nd, Dillard DH. Determination of cardiac output by thermodilution during hypothermia. Am J Cardiol 1980;46:419-22.

[81] Kissling G, Ross C, Brändle M. Validity of thermal dilution techique for measurement of cardiac output in rats. Am J Physiol 1993;265:H-1007-13.

[82] Lin YC, Dawson CA, Horvath SM. The expression of cardiac output in the albino rat, Rattus rattus. Comp Biochem Physiol 1970;33:901-9.

[83] Ashwood ER, Kost G, Kenny M. Temperature correction of blood-gas and pH measurements. Clinical Chemistry 1983;29:1877-85.

[84] Tani M, Neely JR. Role of intracellular Na+ in Ca2+ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Circ res 1989;65:1045-56.

[85] Tani M, Neely JR. Na+ accumulation increases Ca2+ overload and impairs function in anoxic rat heart. J Mol Cell Cardiol 1990;22:57-72.

[86] Kao JPY, Li G, Auston DA. Practical aspects of measuring intracellular calcium signals with flourescent indicators. Methods Cell Biol 2010;99:225-61.

[87] Walker JM. Methods in molecular biology. In: 1999.

[88] Smith G, Reynolds M, Burton F, Kemi OJ. Confocal and multiphoton imaging of intracellular Ca2+. Methods Cell Biol 2010;99:225-61.

[89] thorlabs.de. Laser scannig microscopy tutorial. 2013.
Ref Type: Online Source

[90] Invitrogen MP. The handbook: A guide to fluorescent probes and labeling technologies. 2005.

[91] Dedkova EN, Blatter LA. Measuring mitochondrial function in intact cardiac myocytes. J Mol Cell Cardiol 2012;52:48-61.

[92] Takahashi A, Camacho P, Lechleiter JD, Herman B. Measurement of intracellular calcium. Physiol Rev 1999;79:1089-125.

[93] Lakowicz JR. Principles of fluorescence spectroscopy. 2006.

[94] Thomas AP, Delaville F. Fluorescent Ca2+ indicators. In: McCormack JG, Cobbold PH, editors. Cellular calcium; A practical aproach. New York: Oxford University Press; 1991.

[95] Ytrehus K, Aspang EM. Phospholipid peroxidation in isolated perfused rat hearts subjected to hypothermia followed by rewarming: inverse relation to loss of function. Cryobiology 1994;31:263-71.

[96] Popovic V. Survival time of hypothermic white rats (15 degrees C) and ground squirrels (10 degrees C). Am J Physiol 1960;199:463-6.

[97] Puglisi JL, Yuan W, Bassani JW, Bers DM. Ca(2+) influx through Ca(2+) channels in rabbit ventricular myocytes during action potential clamp: influence of temperature. Circ res 1999;85:e7-e16.

[98] Bjørnstad H, Tande PM, Refsum H. Mechanisms for hypothermia-induced increase in contractile force studied by mechanical restitution and post-rest contraction in guinea-pig papillary muscle. Acta Physiol Scand 1993;148:253-64.

[99] Aasum E, Lathorp DA, Henden T, Sundset R, Larsen TS. The role of glycosis in myocardial calcium control. J Mol Cell Cardiol 1998;30:1703-12.

[100] Gambassi G, Cerbai E, Pahor M, Capogrossi MC, Carbonin P, Mugelli A. Temperature modulates calcium homeostasis and ventricular arrhythmias in myocardial preparations. Cardiovasc Res 1994;28:391-9.

[101] Groban L, Zapata- Sudo G, Lin M, Nelson TE. Effects of moderate and deep hypothermia on Ca2+ signaling in rat ventricular myocytes. Cell Physiol Biochem 2002;12:110.

[102] Schiffmann H, Gleiss J, von Hrischeydt A, Schröder T, Kahles H, Hellige G. Effects of epinephrine on the myocardial performance and haemodynamics of the isolated rat heart during moderate hypothermia -importance of calcium homeostasis. Resuscitation 2001;50:309-17.

[103] Stowe DF, Boban M, Kampine JP, Bosnjak ZJ. Reperfusion with adenosine and nitroprusside improves preservation of isolated guinea pig hearts after 22 hours of cold perfusion with 2,3 butanedione monoxime. J Cardiovasc Farmacol 1993;21:578-86.

[104] Stowe DF, Habazettl H, Graf BM, Kampine JP, Bosnjak ZJ. One-day hypothermic preservation of isolated hearts with halothane improves cardiac function better than low calcium. Anesthesiology 1995;83:1065-77.

[105] Bers DM. Calcium cycling and signaling in cardiac myocytes. Annu Rev Physiol 2008;70:23-49.

[106] Lee HC, Mohabir R, Smith N, Franz MR, Clusin WT. Effect of ischemia on calcium-dependent fluorescence transients in rabbit hearts containing indo 1. Correlation with monophasic action potentials and contraction. Circulation 1988;78:1047-59.

[107] Carafoli E. The fateful encounter of mitochondria with calcium: how did it happen? Biochimica et Biophysica Acta 2010;1797:595-606.

[108] Crompton M. The mitochondrial permeability transistion pore and its role in cell death. Biochem J 1999;341:233-49.

[109] Levine B, Yuan J. Autophagy in cell death: an innocent convict? J Clin Invest 2005;115:2679-88.

[110] Maack C, O'Rourke B. Excitation-contraction coupling and mitochondrial energetics. Basic Res Cardiol 2007;102:369-92.

[111] McCormack JG, Halestrap AP, Denton RM. Role of calcim ions in regulation of mammalian intramitochondrial metabolism. Physiological Reviews 1990;70:391-425.

[112] Tarasov A, Griffiths EJ, Rutter G. Regulation of ATP production by mitochondrial Ca(2+). Cell Calcium 2012;52:28-35.

[113] Wold RM, Kondratiev T, veita T. Myocardial calcium overload during graded hypothermia and after rewarming in an in vivo rat model. Acta Physiol 2012;207:460-9.

[114] Delmotte P, Yang B, Thompson MA, Pabelick CM, Prakash YS, Sieck GC. Inflammation alters regional mitochondrial Ca^{2+} in human airway smooth muscle cells. Am J Physiol Cell Physiol 2012:3-c244.