UiT The Arctic University of Norway

Faculty of Health Sciences
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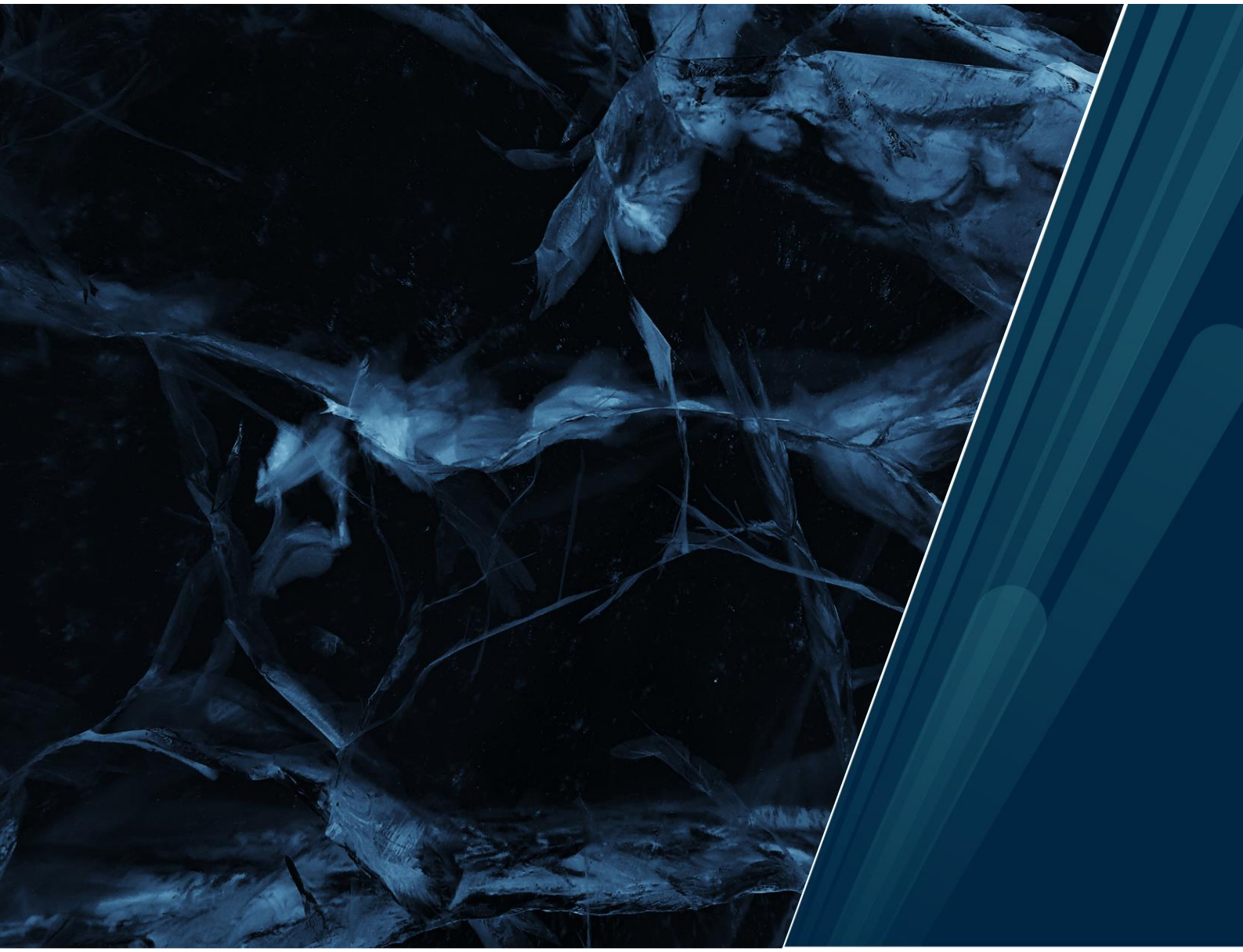
Effects on cellular elimination of cAMP and cGMP

Adrina Kalasho Kuzmiszyn

A dissertation for the degree of Philosophiae Doctor – June 2023



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1 Acknowledgements

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2 Abbreviations

$[Ca^{2+}]_i$:	Intracellular calcium concentration
cAMP:	Cyclic adenosine monophosphate
cGMP:	Cyclic guanosine monophosphate
cTnI:	Cardiac troponin I
CI:	Cardiac index
CO:	Cardiac output
CPB:	Cardiopulmonary bypass
CPR:	Cardiopulmonary resuscitation
ECLS:	Extracorporeal life support
ECMO:	Extracorporeal membrane oxygenation
EDTA:	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER:	Emergency room
HCD:	Hypothermia-induced cardiac dysfunction
HR:	Heart rate
ICU	Intensive care unit
IOV	Inside-out vesicle
KRPB/G	Krebs-Ringer-Phosphate-Buffer containing glucose
MAP	Mean arterial pressure
SNP:	Sodium nitroprusside
SR:	Sarcoplasmic reticulum
SV:	Stroke volume
SVR:	Systemic vascular resistance
SVRI:	Systemic vascular resistance index
VF:	Ventricular fibrillation

3 List of Papers

The included papers in this thesis are arranged by theoretical relevance and will be referred to by their appointed number.

I. Kuzmiszyn AK, Selli AL, Furuholmen M, Smaglyukova N, Kondratiev T, Fuskevåg OM, Sager G, Dietrichs ES. Moderate but not severe hypothermia increases intracellular cyclic AMP through preserved production and reduced elimination. Cryobiology. 2023 Mar;110:18-23.

II. Kuzmiszyn AK, Selli AL, Smaglyukova N, Kondratiev T, Fuskevåg OM, Lyså RA, Ravna AW, Tveita T, Sager G, Dietrichs ES. Treatment of cardiovascular dysfunction with PDE3-inhibitors in moderate and severe hypothermia – Effects on cellular elimination of cyclic adenosine monophosphate and cyclic guanosine monophosphate. Front Physiol. 2022 July;13:923091.

III. Selli AL, Kuzmiszyn AK, Smaglyukova N, Kondratiev T, Fuskevåg OM, Lyså RA, Ravna AW, Tveita T, Sager G, Dietrichs ES. Treatment of cardiovascular dysfunction with PDE5-inhibitors – Temperature dependent effects on transport and metabolism of cAMP and cGMP. Front Physiol. 2021 July;12:695779.

4 Abstract

Background: Accidental hypothermia is associated with high mortality rates. One of the feared complications is hypothermia-induced cardiac dysfunction (HCD) during rewarming. In vivo studies with phosphodiesterase (PDE) inhibitors and sodium nitroprusside (SNP) have demonstrated positive effects on reversing HCD, by both inodilation and vasodilation. Most pharmaceuticals, including PDE inhibitors, act by indirectly increasing intracellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). ATP-binding cassette (ABC) transporters, ABCC4 and ABCC5, have shown to impact cyclic nucleotide levels by cellular expulsion. This thesis aimed to investigate the pharmacodynamic properties of three PDE3 inhibitors (milrinone, amrinone, levosimendan) and two PDE5 inhibitors (sildenafil, vardenafil) in hypothermic settings (paper II, III). The effects of differentiated hypothermia on cellular elimination of cyclic nucleotides were also explored (paper I).

Methods: Embryonic H9c2 cardiac cells were incubated at 37 – 30 – 20°C to determine intracellular baseline cAMP concentrations. Recombinant PDE3 and PDE5, as well as ABCC4 and ABCC5 were incubated at 37 – 34 – 32 – 28 – 24 – 20°C, to determine activity. The effects of PDE inhibitors were assessed by using the same enzymes, as well as inverted erythrocytes to quantify enzymatic breakdown and cellular expulsion of cyclic nucleotides at all six temperatures. Regular erythrocytes were applied to determine the degree of cellular access of each drug at the same temperatures. The resulting dose-response curves provided the foundation for calculating IC_{50} and K_i values.

Results: cAMP was significantly increased in H9c2 cells at 30°C, but not at 20°C. Activity levels of PDEs and ABC transporters were determined at 37°C – 20°C. All drugs were able to cross the cell membrane during hypothermia. IC_{50} and K_i values for the ability of milrinone to

hinder cGMP efflux were significantly lower at 24°C and 20°C, compared to normothermia. Sildenafil IC_{50} and K_i values increased significantly for enzymatic breakdown of cGMP at 32°C, 24°C and 20°C, as well as enzymatic breakdown of cAMP at 20°C. There were no alterations in the potency of the remaining drugs in hypothermia compared to the normothermic controls.

Conclusion: Cyclic nucleotide elimination by PDEs and ABC transporters exhibited temperature-dependent reductions in activity. Both PDE3 and PDE5 inhibitors were able to inhibit cyclic nucleotide elimination through enzyme and transporter suppression below 30°C. However, as these proteins exhibited temperature-dependent reduction in activity, there is a need for temperature-adjusted dosage reduction in future potential treatment algorithms.

5 Introduction

5.1 Definition and classification of hypothermia

5.1.1 Accidental hypothermia

Accidental hypothermia is defined as an unintentional drop in body core temperature below 35°C (1). Low core temperature has a crucial impact on all physiological processes in the human body and the extent of the pathophysiology is determined by the depth and duration of hypothermia. The European Resuscitation Council classifies hypothermia into three different categories based on temperature: mild accidental hypothermia (I: 35 - 32°C), moderate accidental hypothermia (II: 32 - 28°C), and severe accidental hypothermia (III: <28°C) (2). The Swiss staging system uses clinical signs to categorize severity into five different stages; **I** clear consciousness with shivering, **II** impaired consciousness without shivering, **III** Unconsciousness, **IV** apparent death, and **V** death due to irreversible hypothermia. A revised version of the staging system has been proposed, which uses the risk of cardiac arrest to determine the stage (1). A distinction is typically made between primary accidental hypothermia (the inability to maintain core temperature above 35°C solely due to environmental cooling) and secondary accidental hypothermia (hypothermia caused by underlying pathology such as disease, trauma, drugs, etc.) (3).

5.1.2 Therapeutic hypothermia

Controlled induced hypothermia decreases the metabolic rate, including the cerebral metabolic rate, which is shown to have a protective effect in anoxic brain injury. The cerebral metabolic rate decreases as the temperature drops, which mitigates the adverse cellular

reactions to ischemic events (4–6). Consequently, treatment with therapeutic hypothermia (32-36°C) was previously recommended by both the American Heart Association and the European Resuscitation Council for comatose post-cardiac arrest patients for 12-24 h (7). However, after two larger randomized clinical trials failed to show any benefits of therapeutic hypothermia (33°C) in this patient group, the European guidelines concluded that there was not enough evidence, neither to advocate nor dismiss induced hypothermia as neuroprotective treatment. Instead, active fever prevention is recommended (≤ 37.7°C) for 72 h (8–10).

Moreover, therapeutic hypothermia remains an important strategy for neuroprotection in cardiac, aortic and intracranial surgery (7). In aortic arch surgery, deep hypothermic circulatory arrest has been the conventional mode to provide a neuroprotective strategy, due to reduced cerebral metabolism. It has, however, been associated with complications such as neuronal damage and increased organ dysfunction due to endothelial dysfunction (11). It is generally considered safe to apply deep hypothermic circulatory arrest for 30-40 minutes, longer time periods should be avoided to prevent severe neurological complications (12). Alternatives have continuously been explored in order to improve outcomes postoperatively. As improved surgical strategies have emerged, such as antegrade cerebral perfusion, the simultaneous application of moderate hypothermic circulatory arrest has been shown to significantly reduce mortality and stroke rates, as concluded by a recent meta-analysis. Shorter cardiopulmonary bypass (CPB) times were also evident. There were less respiratory complications and lower rates of sepsis, as depicted by shorter stays in intensive care units (ICU) (13).

5.2 Epidemiology

Death caused by cooling has been acknowledged since ancient times (14). Contrary to popular belief, accidental hypothermia is not only seen in circumpolar areas but also in warmer climate zones and can affect anyone given the right circumstances. As such, studies that have tried to estimate the incidence rates in different populations, report varying results. It is evident that underreporting of hypothermia in patients, due to failure to detect it, is an important issue. A retrospective study from Poland has tried to highlight this underestimation. Accidental hypothermia was officially diagnosed in 1.26 per 100 000 inhabitants in Poland in 2011, but following data analysis of questionnaires from polish emergency rooms (ERs), the actual incidence most probably exceeded the official records by up to four times, estimating up to 5.05 cases per 100 000 residents per year (15). The majority of these patients were admitted with secondary accidental hypothermia, due to the combination of cold air exposure and alcohol intoxication, followed by cooling caused by cold air exposure or cold-water immersion. Poor social living conditions were seen in 25% of the patients and approximately 40% were homeless. Denmark exhibited similar incidence rates, averaging overall 4.4 cases per 100 000 residents annually. Not surprisingly, more patients were admitted with accidental hypothermia during winter months, compared to the rest of the year, and the majority were diagnosed with primary accidental hypothermia (16).

Accidental hypothermia is commonly seen in trauma patients where hypothermia acts as an independent risk factor for increased mortality rate. Trauma patients are inherently at risk for developing hypothermia because of exposure to cold environments, deranged homeostasis, including impaired coagulation haemostasis, and volume resuscitation with cool products in case of profound bleeding. Ireland et al reported retrospectively that 13.25% of all major trauma patients admitted to a level I trauma centre in Australia were hypothermic (17). A

German study documented core body temperatures below 36°C in 37% of the multiply injured patients presenting in domestic ERs. A concomitant drop was observed in the Glasgow coma scale and systolic blood pressure. Lower core temperatures were also associated with higher injury severity scores (18).

Accidental hypothermia is associated with a high mortality rate, both in-hospital and during the first year after admission, ranging from 22-43%. Mortality rates vary depending on the type of accidental hypothermia, being highest in hypothermic trauma patients, followed by secondary and primary accidental hypothermia (16,19–22) .

5.3 Cardiovascular pathophysiology of hypothermia

5.3.1 Hypothermia-induced cardiac dysfunction

Hypothermia-induced cardiac dysfunction (HCD) is a feared complication, commonly seen in deeply hypothermic patients during active rewarming in the hospital setting. This condition is characterized by a persisting left ventricular dysfunction during and after rewarming, which is accompanied by low arterial blood pressure without any evident arrhythmias (23). If left untreated, so called *rewarming shock* will follow (24).

The underlying pathophysiology has partially been explained. Ex vivo studies, involving excised rat hearts, showed that deep hypothermia (<15°C) caused more than a six-fold increase in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in cardiomyocytes (25,26) and subsequent rewarming resulted in a 33% reduction, although the concentrations did not return to baseline values, as observed in the normothermic controls (25). Concomitant physiological measurements exhibited significantly lower stroke volume (SV) and cardiac output (CO) following rewarming, compared to the control group, indicating that impaired intracellular

calcium metabolism could contribute to HCD (26). However, preserving intravenous blood volume by either administrating saline or dextran early during rewarming, alleviated cardiac dysfunction with restoration of CO and SV once back in normothermic conditions. Systemic vascular resistance (SVR) did on the other hand remain largely unaltered, compared to the control group – showcasing a need for additional treatment beside fluid therapy to completely restore circulatory function back to pre-hypothermic status. The post-hypothermic increase in myocardial $[Ca^{2+}]_i$ was reduced in this study as a consequence of fluid resuscitation but failed to return to baseline levels after rewarming (27).

Several basic research experiments involving rats and rat cardiomyocytes, have however demonstrated that profound hypothermia (<15°C) and subsequent rewarming cause a decrease in intracellular Ca^{2+} sensitivity and increased cardiac troponin I (cTnI) phosphorylation, leading to a disruption in the excitation-contraction coupling (28,29). In contrast, mild (34°C) and moderate (32-30°C) hypothermia have proved to increase both rat and guinea pig ventricular myocyte contractility and simultaneously cause higher intracellular Ca^{2+} transients (30,31). Increased contractility is even persistent in guinea pig cardiomyocytes in severe hypothermia (22°C). Furthermore, Ca^{2+} transients and diastolic Ca^{2+} concentrations are significantly higher in the hypothermic cells, and both the sarcoplasmic reticulum (SR) Ca^{2+} storage and fractional SR Ca^{2+} releases are increased (32).

These conflicting results, showing both positive and negative inotropic effects of hypothermia, could be explained by inter-species differences, different hypothermia protocols and the limitations associated with evaluating cardiac function in an ex vivo model. It has also been hypothesized that hypothermia induces cardiac dysfunction by exaggerated formation of reactive oxygen species, which ultimately disrupt excitation-contraction coupling through mechanisms described above. This is supported by that antioxidant treatment (TEMPOL)

seems to mitigate cTnI phosphorylation, exhibiting promising results for treating cardiac dysfunction in hypothermia (33).

5.3.2 Cardiac electrophysiology in hypothermia

Hypothermia is known to affect cardiac electrophysiology, and the risk of cardiac arrest increases as the body core temperature progressively drops (2,34,35). The risk of atrial fibrillation and atrioventricular block increases, including total block, as well as the risk of ventricular fibrillation (VF) (36,37). Curiously, the risk of arrhythmias appears increased primarily in moderate (31°C) hypothermia - an effect which is explained by a prolonged repolarization phase. In contrary, severe (17°C) hypothermia cause changes in both ventricular depolarization and repolarization, which are shown to be anti-arrhythmic (38).

Under normothermic conditions, many different pharmaceuticals are known to cause various arrhythmias. Inotropic agents that specifically stimulate β-receptors, such as dobutamine, dopamine, and epinephrine, have shown to either cause or exacerbate atrial fibrillation and flutter. Dobutamine has the potential of also inducing or aggravating several other arrhythmias – atrial tachycardia, atrioventricular node reentrant tachycardia and monomorphic ventricular tachycardia (39). Naturally, similar proarrhythmic attributes would be expected in hypothermia.

A previous case report describes how a healthy 36-year-old man was brought to the ER, after a witnessed out-of-hospital cardiac arrest with asystole. He was treated conventionally with cardiopulmonary resuscitation (CPR) and received 2 mg adrenaline and 2 mg atropine, intravenously. Spontaneous circulation returned, with sinus tachycardia, but the patient remained unconscious. He was subsequently intubated and received lidocaine infusion before

therapeutic hypothermia was induced (33.5°C). Shortly after transferal to the ICU and commencing sedation, the patient had recurring episodes of VF. Epinephrine-, dobutamine-, and dopamine-infusions were started simultaneously. Extracorporeal membrane oxygenation (ECMO) was initiated, and a bedside echocardiogram showed acute profound heart failure. Due to almost persisting VF, the patient received 122 (!) defibrillations, as well as 4.4 mg of epinephrine, 164 mg of dobutamine, 120 mg dopamine, amongst other antiarrhythmic medications. The patient was transferred to another hospital after 12 h for potential heart transplantation. The receiving physicians suggested that the refractory VF could be caused by either the substantial dosages of medications or therapeutic hypothermia. Hence, all vasoactive agents were halted, and the patient was rewarmed. A single defibrillation converted the patient to sinus rhythm and on day three the mechanical circulatory support and ventilator were withdrawn. The patient had initially impaired short-term memory, but full neurological recovery was evident after 10 days. He received an automatic implantable cardioverter defibrillator before he could be discharged (40).

Arrhythmia is not uncommon in comatose post-cardiac arrest patients subjected to therapeutic hypothermia, as illustrated in a recent publication by Dankiewicz et al. In this open-label, randomized trial patients were allocated to either undergoing targeted hypothermia (33°C) or early prevention of fever (defined as temperatures $\geq$ 37.8°C). The incidence of hemodynamically compromising arrhythmia was 24% in the hypothermia group compared to 17% in the control group. There were no differences in other adverse events between the groups (10).

However, the combined application of hypothermia and cardioactive drugs does not necessarily lead to hemodynamically significant arrhythmia. In hypothermic swine models (35°C, 30°C), administering either dobutamine- or dopamine-infusions did not cause any arrhythmias (41). Likewise, adrenaline-infusion in hypothermic pigs (32°C) has not been

shown to cause any cardiac arrhythmias (42). An experiment, using ex vivo spontaneously beating rabbit hearts, did not manage to induce any irregularities in the cardiac function during cooling to 22°C and administrating either adrenaline, dobutamine, or isoprenaline (43).

Evidently, post-cardiac arrest patients could hardly be directly compared to the intact animal hearts in the beforementioned studies. In the case report by Poles et al. (40), three different inotropic medications with similar pharmacological mechanisms (all increasing myocardial cAMP levels) were initiated simultaneously, possibly being more harmful than therapeutic for the patient.

5.3.3 Vascular function and intravascular hemodynamics in hypothermia

During hypothermia, increased SVR is evident, although the sympathetic cardiovascular control seems to be weakened (44). Rewarming back to normal core body temperature does not reverse this alteration (45,46). Furthermore, surface cooling to severe hypothermia (27.8 ± 1.6°C) induces extravasation of both plasma and serum proteins, indicating a concomitant affection of the microcirculation, which leads to an increased hematocrit and interstitial oedema (47,48). Blood viscosity increases and the deformability of erythrocytes and other cells in the blood becomes impaired in cold temperatures which further aggravates the hypothermia-induced microcirculatory dysfunction (47,49).

Investigating the microcirculation in Syrian golden hamsters, profound hypothermia (18°C) during extracorporeal circulation led to reduced functional capillary density and increased microvascular permeability during the first hour of the experiment. While the former was completely restored after rewarming, the extent of oedema progressively increased (50). In

the control group (normothermia + extracorporeal circulation), a similar grade of extravasation was observed.

Additionally, the phenomenon of *cold diuresis*, which occurs during exposure to cold temperatures, is seen in relation to systemic hemodynamic changes (47,51). Reduction of core temperature to 25°C in rats causes a profound drop in the glomerular filtration and nephron reabsorption rate, as well as increased secretion of sodium in the urine, further contributing to excessive diuresis (52).

5.4 Treatment of accidental hypothermia

5.4.1 General

International and national guidelines for treating hypothermic patients take the degree of cooling and the state of consciousness into consideration. An important distinction has also been made between treating the patient in the pre-hospital and in-hospital settings.

In general, it is important to prevent further heat loss in the pre-hospital milieu by promptly extricating and transporting patients to the nearest hospital. However, awake patients with mild hypothermia can often be treated on site with passive rewarming – wrapping with various blankets or drapes to provide insulation –, offerings of warm beverages and encouragement to move actively before being admitted to the hospital is advised. Moderately and severely cooled patients require active rewarming besides packing with several layers of wrappings (2).

Active rewarming outside of the hospital entails external and minimally invasive treatment options, such as electric and chemistry blankets, heating pads and warmed intravenous fluids.

Moreover, it is recommended, under these circumstances, that the patients are placed in a horizontal position during transportation to prevent arrhythmias and *rescue collapse* – witnessed cardiac arrest in hypothermic patients. Drenched clothes should be removed in a controlled environment, typically in an ambulance, provided that insulating layers are available. If not possible, a vapor barrier should be added over the wet clothing (2,53,54). Patients that are at high risk of going into cardiac arrest (core temperature <30°C, ventricular arrhythmia, sBP <90 mmHg) and patients that are already in arrest, are transported during continuous CPR to an extracorporeal life support (ECLS) centre for rewarming (4).

5.4.2 Cardiac arrest

CPR management in hypothermic settings is similar to the treatment in normothermia but with some differences. Adrenaline is not administered if the core temperature is <30°C, and the interval for administrating the drug is increased to 6-10 minutes (instead of 3-5 minutes) if the core temperature is <36°C, according to the European guidelines (4). The rational for withholding adrenaline is based on a few animal studies. In hypothermic cardiac arrest models using pigs (28°C and 26°C), administrating intermittent adrenaline showed an increase in the coronary perfusion pressure, compared to placebo. However, there were no differences in the incidence of return of spontaneous circulation (55,56). In pigs receiving placebo, the coronary perfusion pressure was estimated to ~ 15 mmHg, which is deemed the therapeutic cutoff for continued resuscitation (56,57). Due to the lacking evidence of survival in temperatures <30°C and the risk of myocardial injury with adrenaline injections, it was concluded that this medication should be withheld until the core temperature reaches ≥ 30°C (4).

Recommendations for defibrillation during rewarming, state that if VF remains after three rounds of shock therapy, further attempts should be delayed until the core temperature reaches

30°C. In the pre-hospital setting, delayed and intermittent CPR might be practiced in hypothermic arrested patients (<28°C) due to complicated evacuation and transportation to nearest hospital. Intermittent CPR constitutes typically of five minutes of uninterrupted CPR, followed by 5-10 minutes of transportation until the evacuation has been successful (4,58).

Rewarming in-hospital should be managed with ECLS if a suited center can be reached within six hours. Otherwise, other measures will be employed to rewarm the patients, such as thoracic or peritoneal lavage, continuous renal replacement therapy, hemodialysis, intravascular catheter rewarming with warm fluids, amongst others (2,4). Although these methods are not as effective and safe to use in hypothermia as ECLS, they do provide alternatives in areas where access to advanced health care facilities are unavailable or difficult to reach. As illustrated in a recent case report, a 57-year-old man with unwitnessed out-of-hospital cardiac arrest and a core temperature of 23°C in midwinter Wisconsin, was successfully resuscitated by using these conventional rewarming methods simultaneously with prolonged CPR (4 h 56 minutes). He had a full neurological recovery the day after (59).

In general, it is recommended to use HOPE or ICE scores to determine prognosis and whether ECLS treatment has potential to provide successful resuscitation or not. HOPE stands for **H**ypothermia **O**utcome **P**rediction after **E**CLS and is based on six variables that are available at hospital admission: age, sex, mechanism of hypothermia, core temperature (preferably in-hospital), serum potassium value, and CPR duration. Similarly, ICE score includes gender, +/- asphyxiation, and serum potassium concentration to predict survival in hypothermic patients requiring ECLS. The use of only in-hospital serum potassium and core temperature in the decision making is less reliable (4,60,61).

5.4.3 Rewarming shock

The treatment of rewarming shock shares similarities to the treatment algorithm of hypothermic cardiac arrest in the national and regional guidelines in Norway. Circulatory failure during rewarming is routinely treated with warm, physiological fluids intravenously, to replace the loss of fluid by cold diuresis and extravasation. Ringer-Acetat is the first choice of fluids, to reduce the risk of inducing metabolic acidosis. If the sBP fails to reach at least 90 mmHg, Northern-Norwegian guidelines endorse the use of low to moderate doses of dopamine to support the circulation (58,62). However, both the national and the European guidelines do not advocate this regimen. Persisting circulatory instability is an indication for commencing ECLS. (4,53).

5.5 Cardiovascular support during therapeutic hypothermia

In contrast to accidental hypothermia, therapeutic hypothermia is achieved under controlled circumstances. As such, it generates a little less challenging hemodynamic situation and is monitored closely through the whole phase. The risk for complications in the ICU is highest during cooling and rewarming.

In the induction phase, when the patient is cooled to mild or moderate level of hypothermia (>30°C), there is a high risk for developing hypovolemia due to cold-induced diuresis. Short-term management with fluid administration and use of vasoactive drugs is warranted (63). Naturally, both the HR and CO start to drop while the blood pressure slight increases due to increased SVR. Treatment is often not required as the balance between the metabolic supply and demand is maintained. In this phase there is also risk for developing hypokalemia, hypophosphatemia, and hypomagnesemia due to intracellular shift of electrolytes and renal

loss, besides hypothermia-induced ECG changes. Thus, the risk of arrhythmias increase as the temperature declines. Replacement of electrolytes and reducing the risk of complications by shortening the cooling phase is often considered (64).

During the maintenance phase of therapeutic hypothermia, the patient is generally hemodynamically stable if potential issues from the induction phase have been addressed.

In therapeutic hypothermia it is generally considered that rapid rewarming should be avoided as there are higher risk acquiring hyperkalemia, thought to be caused by release of intracellular potassium to the bloodstream. The risk is especially increased in patients with renal failure. Hence, the recommended rewarming rate is 0.2-0.5°C per hour (63,64).

5.6 Pharmacological support during hypothermia

5.6.1 Cyclic nucleotide regulation for cardiovascular support

Cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are important intracellular second messengers, involved in numerous processes, including regulating cardiovascular function (65).

The intracellular levels of cAMP and cGMP are precisely regulated by the rate of synthesis by adenylyl cyclase (AC) and guanylyl cyclase (GC) and the rate of degradation carried out by phosphodiesterases (PDEs) (66,67). Additionally, active cellular efflux through membrane-bound ATP-binding cassette (ABC) transporters help to keep the intracellular concentrations of cyclic nucleotides within the physiological ranges (68,69).

There are several isoforms of PDEs that are expressed in the human physiology but not all have been reported in the cardiovascular system. PDE1-5 and PDE8-10 are expressed in the

heart; PDE1-3 and PDE10 hydrolyze both cyclic nucleotides but with varying affinities, PDE4 and PDE8 hydrolyze cAMP, specifically. PDE5 resides abundantly in the vascular system and metabolizes primarily cGMP (70,71).

The ABC transporter ABCC4 (MRP4), which is present in many organs of the human body, is known for actively transporting cAMP out of the cell with moderate to high affinity. ABCC4 has an additional transport site with high Km for both cAMP and cGMP. Other ABC transporters, such as ABCC5 (MRP5) and ABCC11 (MRP8) both transport cAMP with low affinity. In addition, ABCC5 transports cGMP with high affinity (72).

Several pharmacological approaches have been developed to modulate cyclic nucleotide levels in order to treat various cardiovascular disorders (73). Catecholamines, such as adrenaline and noradrenaline, increase cAMP levels through extracellular β-receptor mediated stimulation of G-protein coupled signaling and activation of AC, while bipyridine derivatives, such as milrinone and amrinone, inhibit PDE3, thereby inhibiting the degradation instead and indirectly causing an increase in intracellular cAMP concentration (74–76). PDE inhibitors sildenafil and vardenafil prevent cGMP hydrolysis by blocking PDE5, thus, increasing the intracellular levels of cGMP (77).

The consecutive physiological effects of higher intracellular levels of cyclic nucleotides are diverse and depending on the cells affected. Increased cAMP levels in the cardiomyocytes lead to higher heart rate (HR; chronotropy), better ability to contract (contractility), and faster myocardial relaxation (lusitropy). The net effect is larger amount of blood pumped by the heart into the circulation, reflected in higher SV and CO (78). In vascular smooth muscle cells (VSMC), higher cAMP and cGMP induce relaxation through different mechanisms. The result is lower SVR (79,80).

5.6.2 Adrenergic agonists

5.6.2.1 Adrenaline

Adrenaline is a potent adrenergic receptor agonist that gives a dose-dependent stimulation of both α- and β-adrenoceptors in the cardiovascular system. Low-dose adrenaline stimulates primarily β-adrenoceptors which results in positive inotropy and chronotropy (β_1/ β_2), as well as vasodilation (β_2). The net effect is often lower BP, both systolic and diastolic. In higher doses, it causes profound arterial and venous vasoconstriction; an effect caused by a predominant α-receptor agonism, which increases BP (76,81–83).

The diverging effects of adrenaline, through stimulation of the different adrenoceptors, depend on the type of G protein that is activated following binding. Stimulatory G protein (Gs) activates AC and thereby increases cAMP in the cell while inhibitory G protein (Gi) inhibits AC. Both β-adrenoceptors are connected to Gs and therefore cause a rise in cAMP levels upon activation in normothermia. α_2-adrenoceptors are coupled to Gi. α_1-adrenoceptors, however, are connected to Gq protein, another subtype of G proteins, which activate another intracellular signaling pathway - the phospholipase C – inositol triphosphate (IP3) pathway – with different downstream reactions that do not include cyclic nucleotides. The resulting reaction is contraction of smooth muscle cells surrounding the blood vessels (76).

The effects of adrenaline in hypothermia have been investigated. Predictably, administrating a high dose of adrenaline (1.25 µg/min) in normothermic rats led to increased SV, HR, and CO. However, the same effects were not seen when cooling the animals down to severe and profound hypothermia (28°C and 15°C), despite demonstrating increased β-adrenoceptor sensitivity (84). Already at 33°C, the inotropic effect of adrenaline seemed to be diminished. In contrast, low dose adrenaline (0.125 µg/min) increased HR and CO, but not mean arterial pressure (MAP), during cooling to 30°C. These positive effects were not apparent at

temperatures below 30°C (85). Similarly, in rewarmed rats (15 → 37°C), high-dose adrenaline-infusion (1.25 μg/min) induced an increase in HR, SV, and CO, although not reaching normothermic values. SVR parameters were significantly elevated in the hypothermic setting during drug infusion but kept low after rewarming (84). In the same study, cardiac tissue from the left ventricle was excised after rewarming and analyzed for its cAMP content. cAMP was significantly higher in the normothermic and hypothermic groups receiving adrenaline compared to the untreated normothermic and hypothermic groups. Adrenaline caused a four-fold increase of cAMP in the hypothermic hearts compared to the hypothermic control group.

In another similar rat model, moderate dose of adrenaline (1 μg/min) during cooling (37 → 28°C) gave significantly higher MAP and SVR than in the control group receiving saline. Continued cooling down to 15°C after discontinuing all infusions, did not induce any other changes between the two groups than increased SV and reduced MAP and SVR at 20°C in the intervention group compared to the control. Interestingly, following rewarming, MAP and CO were markedly lowered in the treatment group (86).

Ex vivo studies with adrenaline administered to isolated rat hearts at 28°C confirmed the negative inotropic effect of adrenaline during hypothermia and linked the results to elevated intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) – higher concentrations impeded the response to the drug and could be indicative of calcium overload (87). Another experiment used adrenaline in spontaneously beating rabbit hearts; intact inotropic and chronotropic properties at 22°C were presented but not at 27°C, after cooling. EC_{50}-values, which are the concentrations needed to produce 50% of the maximum response, were reduced significantly at both temperatures, indicating that there was an increased myocardial sensitivity to the catecholamine (43,88).

Inducing moderate hypothermia (32°C) in a porcine model and administrating adrenaline (30 or 90 ng/kg/min), showed positive inotropic effect with increased CO and SV. 90 ng/kg/min also significantly reduced SVR (42). Positive effects on the cardiac contractile function were also observed in profoundly cooled dogs (22°C) after receiving 1 µg/kg bolus injection of adrenaline (89).

5.6.2.2 Noradrenaline

Noradrenaline is currently the preferred choice of vasopressor (drug that induces vasoconstriction) in patients with septic shock to maintain systemic BP. It binds primarily to α-receptors, but has also affinity for β-receptors, resulting in mainly arteriolar vasoconstriction and some degree of positive inotropy and chronotropy (81,82,90).

This medication exerts its vasoactive effects mainly through Gq-coupled α_1-receptor activation which induces vasoconstriction without involving the cAMP-PKA pathway. Noradrenaline also has a high affinity for α_2-receptors in the heart, which limit cAMP production and cause vasodilation in the coronary arteries (76). In contrast, it exerts its positive inotropic effect through β_1-adrenoceptors and the cAMP-PKA pathway (91).

One of the earlier studies conducted with this drug in hypothermia, exhibited positive effects on myocardial contractility in a dog model (core temperature 28-30°C). With higher dosages, a decrease in contractile force was observed instead. Furthermore, using dichloroisoprenaline simultaneously, a β1- and β2-blocker, lead to interruption of both the positive and negative inotropic responses, demonstrating that noradrenaline had maintained affinity for β-receptors in hypothermia (92). In a cat model, administrating moderate- and high-dose noradrenaline-infusion during normothermia caused significantly increased MAP and SVR, but not CO.

However, cooling to the core temperature of 30°C and subsequent rewarming, showed abolished effect on SVR while MAP was maintained. After rewarming, an increased dose of noradrenaline was needed to significantly raise MAP and there was an unsignificant elevation of CO (93). This finding was not reproduced in a later study conducted by the same group, when noradrenaline was administered after rewarming. Instead, CO was increased significantly with moderate and high dose noradrenaline, while there were no effects on MAP and SVR (94).

5.6.2.3 Dobutamine

Dobutamine is a potent β_1-agonist, commonly used in patients with cardiogenic shock and severe heart failure (82,95). Besides increasing CO in normothermia, a concomitant decrease in SVR usually occurs during treatment, owing to the weak effect it has on β_2-receptors (96).

Dobutamine normally increases cAMP in the cells by activating Gs proteins (97,98). It seems that dobutamine and its intracellular downstream reactions, including cAMP levels, have not been investigated in hypothermia.

Riishede and Nielsen-Kudsk used isolated rabbit hearts in 1989 to determine the effects of dobutamine, amongst other catecholamines, in hypothermia. Contraction velocity was increased at 22°C but reduced when the hearts were cooled to 32°C and 27°C with dobutamine infusion. The contraction amplitude was increased at all the above temperatures (43). It seems that dobutamine exhibits temperature-dependency, as demonstrated in a study conducted by Rieg et al. There was almost no inotropic effect at 31°C in guinea pig ventricular trabeculae, despite high dosages. At temperatures 34°C and 37°C, it was evident that it induced progressively stronger contraction force (31). Comparably, simply cooling pigs

from 37°C to 33°C increased the left ventricular contractility to a similar extent as administrating dobutamine to a normothermic porcine heart, which could explain the lack of positive inotropy with the drug in hypothermia, as demonstrated by Rieg et al. (31,99).

5.6.2.4 Isoprenaline

Isoprenaline is a pure non-selective β-agonist that improves cardiac function, reflected in increased SV and CO values. It causes vasodilation and lowered SVR (82,100). Moreover, it also has a positive chronotropic effect, albeit not as prominent as the inotropic response, which is why it is used in the acute management of bradycardia (101).

In normothermia, isoprenaline stimulates an increase in cAMP levels, but fails to do so at 22°C and 7°C in ventricular tissue slices of hamster heart, although its inotropic and chronotropic feature was maintained in isolated rabbit hearts at 22°C (43,102). On the contrary, the inotropic effect of isoprenaline was lacking in hypothermic dogs (25°C), while it was able to increase HR and decrease SVR (103). In vitro studies confirmed this reduced effect at 28°C in rat left atrial preparations, and went further to demonstrate a direct negative inotropic effect at 20°C (104). Thus, the research findings are inconsistent, which may be due to species variation and/or differing experimental protocols.

5.6.2.5 Dopamine

Dopamine is a catecholamine and the immediate precursor to adrenaline and noradrenaline. It exerts its actions in a dose-dependent manner through both adrenergic and dopaminergic receptor activation (81,82). At low doses, dopamine acts on the dopaminergic receptors, which are located on smooth muscle cells in various arteries, such as the renal, mesenteric,

and coronary arteries. The resulting vasodilation does not cause a significant drop in blood pressure (105,106). Increasing the dose leads to β-adrenoceptor activation with increased inotropy and chronotropy, due to the activation of AC and the cAMP-PKA route. Further increase in dosage activates α-adrenoceptors which results in vasoconstriction and increased SVR through phospholipase C and IP3 activation (76).

Although dopamine is not commonly used in the critical care setting, it is the preferred medication for treating hypothermia-induced cardiovascular dysfunction in northern Norway (58). These recommendations are based on several animal studies, albeit with inconsistent results. Dopamine, administered in cooled pigs with core temperature of 30°C and following rewarming, has been shown to improve CO without any risk of inducing hypothermia (41). In cooled pigs (25°C), low-dose dopamine-infusion, reduced SVR index (SVRI; SVR adjusted for body surface area) significantly, while high-dose administration increased both SVRI and MAP without increasing cardiac index (CI; CO adjusted for body surface area). At this temperature, plasma concentrations of the drug were increased by four times compared to the same infusion rate in normothermia and the half-time was doubled. During subsequent rewarming (30-34°C), CI increased due to simultaneous increase in HR with high-dose infusion (62). In another study, low-dose dopamine-infusion in cooled pigs (32°C) showed no significant changes in CO or MAP but high-dose resulted in reduced MAP while CO was unaffected (107).

5.6.3 Nitroprusside

Sodium nitroprusside (SNP) is a potent vasodilator that has been on the market for several decades. SNP is used for hypertensive emergencies and acute heart failure and acts primarily by relaxing the smooth muscle cells surrounding blood vessels (108,109). By releasing nitric

oxide and activating the cGMP-pathway, instantaneous arterial and venous vasodilation are evident (110). In the cardiomyocyte, nitric oxide has a biphasic effect on the contractility. In lower doses, it produces positive inotropy, while inducing a negative inotropic effect in higher concentrations (111,112).

The pharmacological effects of SNP have been explored in hypothermic rodent models. Administrating SNP during severe hypothermia (<15°C) and subsequent rewarming, showed intact vasodilator properties with reduced SVR. Furthermore, positive effects on both CO and SV were seen after rewarming, although the parameters were significantly lower compared to the baseline levels (45). In another animal study with the same experimental setup, SNP was able to increase organ blood flow and, more importantly, the brain blood flow, demonstrating that both the increased SVR and resulting organ dysfunction can be reversed during rewarming and after (113).

Despite the beneficial effect on cardiovascular function during hypothermia, the use of SNP is associated with various risks in normothermia. It is effective in reducing the blood pressure, and sometimes excessively so, causing profound hypotension and increased risk of irreversible ischemic injuries (108).

5.6.4 PDE inhibitors

5.6.4.1 Milrinone

Milrinone is a PDE3 inhibitor and is widely used in clinical settings for treating heart failure in normothermia. By inhibiting PDE3, the enzymatic breakdown of cAMP is impeded which leads to increased cAMP levels in normothermia. Thus, positive inotropy and vasodilation follow (74).

Janelle et al. measured intracellular cAMP concentrations after milrinone administration by randomizing 20 patients undergoing coronary artery bypass, to either receive a bolus of the drug or saline after being placed on CPB and having obtained transmyocardial biopsies. All patients were cooled and kept in moderate hypothermia (28°C), as per standard protocol. When the surgical procedure on the heart was completed and the patients were normothermic again, prior to the discontinuation of the heart-lung machine, another set of biopsies were collected. Subsequent analysis revealed significantly higher cAMP levels in the milrinone-treated group at the end of CPB, compared with placebo (114). There were no demographic differences between the groups, and although the option of treatment was randomized and blinded, the patients in the milrinone group had significantly lower preoperative left ventricular ejection fractions. However, the groups were equally in need of adrenaline and nitroglycerin for cardiovascular support in order to be weaned off the CPB.

Its use in the accidental hypothermic settings has been considered since Tveita et al. reported preserved left ventricular systolic and diastolic function in rats treated with milrinone-infusion both in normothermia but also during cooling to 15°C; an effect that was not observed in the control group, which received saline-infusion. The vasodilator effect of milrinone was absent at low temperatures (115). It was later investigated, by the same research group, whether similar beneficial effects of milrinone-infusion persisted during rewarming in a similar rodent model. The results showed complete restoration of both SV and CO after rewarming (37°C) in the study group, whereas the same variables were significantly reduced in the control group (46). In contrast to the former experiment, total peripheral resistance (TPR) was significantly lower in animals treated with milrinone at 32°C and 37°C (46,115). Rieg et al. compared milrinone to both levosimendan - known as a calcium sensitizer and PDE3 inhibitor in higher dosages, and dobutamine in an in vitro study using guinea pig ventricular trabeculae. The experiment reported reduced inotropic effects of milrinone at 31°C and 34°C, although it

shortened the contraction time – a clear counteractive effect to hypothermia-induced changes (31). As expected, hypothermia had a positive inotropic effect, independent of pharmacological stimulation. In normothermia, the inotropic effects of milrinone were dose-dependent. Levosimendan, on the other hand, was found to be superior to both milrinone and dobutamine at 31°C, and at 34°C, it surpassed milrinone in regard to the increase in contractility.

Milrinone and cilostazol, another PDE3 inhibitor, were used in a previous study to evaluate their potential effects on hypothermia-induced ventricular tachycardia and VF. Gurabi et al. pharmacologically simulated early repolarization patterns, which are seen in Brugada syndrome and various other early repolarization syndromes, by using perfused canine left ventricular wedge preparations. Inducing mild hypothermia (32°C) in these preparations, led to the development of phase two reentry, a local arrhythmogenic phenomenon, and ventricular arrhythmia. Subsequent rewarming to 37°C and the addition of either cilostazol or milrinone, before reducing the temperature to 32°C again, prevented ventricular arrhythmia in five of seven preparations (116).

5.6.4.2 Amrinone

Amrinone, another PDE3 inhibitor, has been applied in a study to accelerate cooling and rewarming rates during neurosurgical procedures through inodilation; giving both positive inotropic effect and reducing SVR (117). Secondary outcomes were the effects on the cardiovascular system. The study group receiving higher doses amrinone, had significantly higher HR and lower MAP during rewarming from the intraoperative temperature (34°C), compared to the control group. The CI tended to be higher in this group, although significant

differences were not found. More prominently, SVR index was significantly lower in the same participants.

Similar findings were evident in a retrospective cohort study when the diastolic arterial pressure was measured in neonates with hypoxic ischemic encephalopathy and concomitant hypoxemic respiratory failure. A sub-group of patients were eligible for therapeutic hypothermia and thus, the body core temperature was maintained at 33-34°C.When inhaled nitric oxide was not sufficient on its own to treat acute pulmonary hypertension, these patients had milrinone added as an adjunct treatment (118). The effects on the cardiovascular system were profound; the diastolic arterial pressure was reduced significantly within the first hour of exposure and the systolic arterial pressure followed the same pattern, albeit not to same extent. Not surprisingly, the group exhibited a higher HR and the demand for vasoactive medications was dramatically increased.

5.6.4.3 Levosimendan

Levosimendan is used to treat decompensated congestive heart failure. It is known as a calcium sensitizer, exerting its pharmacological action through increasing the affinity in Troponin C for ionized calcium and helps stabilize the resulting complex. The result is increased myocardial contraction without affecting intracellular levels of cAMP nor $[Ca^{2+}]_i$ at therapeutic concentrations. In higher concentrations (> 0.3 μM) levosimendan acts as a PDE3 inhibitor, with concomitant increase in cAMP levels. Similar to the other PDE inhibitors, it also functions as a vasodilator by opening ATP-sensitive K^+ channels in smooth muscle cells and myocytes (119).

Levosimendan has been tested in vivo, where it restored SV and CO back to normothermic values after rewarming rats from deep hypothermia (15°C), significantly different from the control group (120). Rungatscher et al. compared the effects of levosimendan to adrenaline in a rodent model after rewarming from deep hypothermic circulatory arrest (13-15°C) and CPB (121). Both drugs exhibited positive effects on the MAP, although adrenaline failed to increase SV, indicating that other factors, such as increased SVR, could explain its effect on MAP – this was, however, not measured. In the same study, levosimendan was able to restore SV to pre-hypothermic values. Furthermore, the levosimendan-treated group exhibited better lusitropic effects and there were no arrhythmic episodes, demonstrating an overall better outcome in this group. The writers confirmed these results in a similar study conducted two years later and simultaneously investigated the metabolic effects of the drugs (122). After rewarming and weaning from CPB, the rats were subjected to treatment with either levosimendan, adrenaline, or saline as control, in normothermia. Subsequent analysis of myocardial biopsies revealed higher levels of intracellular adenosine triphosphate molecules in the former group, compared with the other two. Moreover, the degree of phosphorylation of cTnI was increased five-fold in the control group, compared with pre-hypothermic values. This change was reversed by levosimendan, leading to restoration of physiological status, but was not evident in the adrenaline-treated group.

In patients undergoing mitral valve repair or replacement for mitral stenosis, the hemodynamic effects of levosimendan and dobutamine (an β1-receptor agonist) were compared (123). Similar to the previous study, the surgical protocol included CPB and mild hypothermia (28-32°C). The levosimendan and dobutamine groups were treated with standard infusion rates during the weaning period from CPB and various hemodynamic parameters were recorded for up to 36 hours postoperative. It was evident that levosimendan induced higher grade of vasodilation and accordingly a lower MAP up to 12 hours post CPB. Use of

another inotrope or vasopressor was more frequent in this group. Yet, CI, was significantly higher 24 and 36 hours postoperatively, compared to dobutamine – an effect ascribed to levosimendan's active metabolites. There were no differences in duration of CPB, ventilation or ICU days.

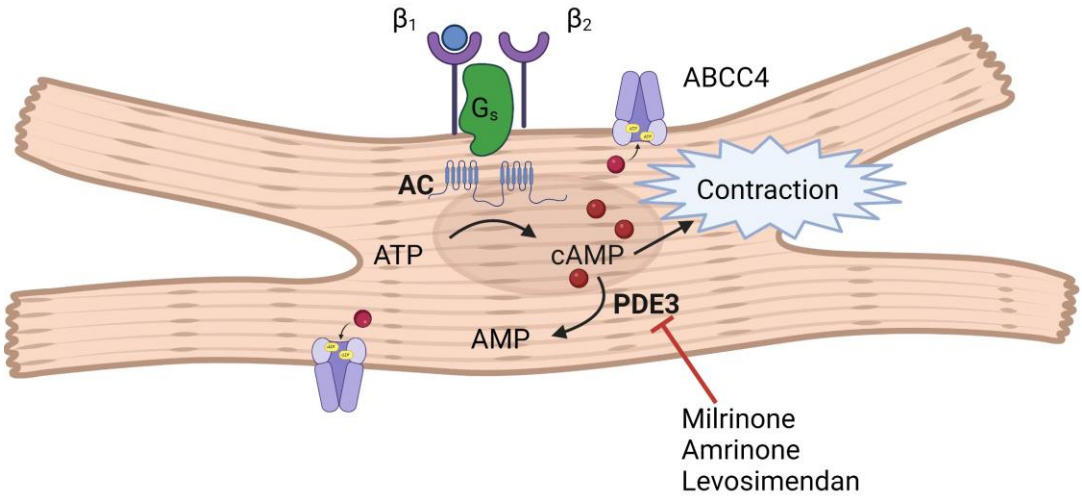


Figure 1. Schematic illustration depicting PDE3 inhibition and cAMP increase, leading to cardiomyocyte contraction. ***PDE3****, phosphodiesterase 3;* ***AC****, adenylyl cyclase;* ***ATP****, adenosine triphosphate;* ***cAMP****, cyclic adenosine monophosphate;* ***AMP****, adenosine monophosphate;* ***ABCC4****, ABC transporter 4;* ***β1/ β2****, adrenoceptors;* ***Gs****, G stimulatory protein.*

5.6.4.4 Sildenafil

Sildenafil is a potent PDE5 inhibitor which effectively raises cGMP levels intracellularly. It was originally developed for the treatment of hypertension and angina pectoris but during the clinical trials it was noted that a recurring side effect was erectile response. Since PDE5 is expressed abundantly in penile corpus cavernosum, sildenafil was the first inhibitor to be licensed for treatment of erectile dysfunction. PDE5 is also abundant in lung vascular smooth muscle, which is why this medication is nowadays used to treat pulmonary arterial hypertension as well (124,125).

When studying hypothermia-dependent effects, sildenafil has to my knowledge only been tested in rat models in the context of neonatal necrotizing enterocolitis, which is outside of the scope of our current project.

5.6.4.5 Vardenafil

Vardenafil is a successor to sildenafil, which is approximately ten times more potent than sildenafil in vitro and has approximately the same elimination half-life as sildenafil (126). Like its precursor, its clinical effects are mediated through increased cGMP levels (127). To my knowledge, it has not been tested previously in hypothermic settings.

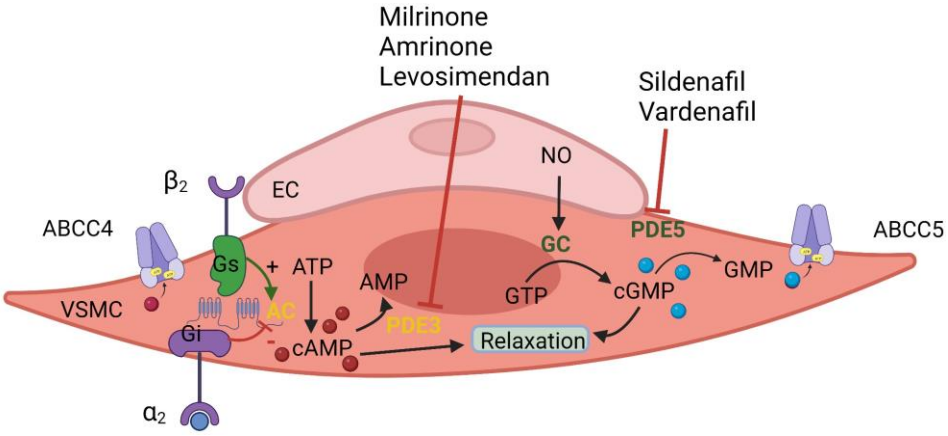


*Figure 2. Schematic illustration depicting PDE inhibition and intracellular cyclic nucleotide increase, inducing vascular smooth muscle cell **(VSMC)** relaxation. **EC**, endothelial cell; **NO**, nitric oxide; **PDE5**, phosphodiesterase 5; **PDE3**, phosphodiesterase 3; **GC**, guanylyl cyclase; **AC**, adenylyl cyclase; **GTP**, guanosine triphosphate; **cGMP**, cyclic guanosine monophosphate; **GMP**, guanosine monophosphate; **ATP**, adenosine triphosphate; **cAMP**, cyclic adenosine monophosphate; **ABCC5**, ABC transporter 5; **ABCC4**, ABC transporter 4; **β1/ β2**, adrenoceptors; **Gs**, G stimulatory protein; **α1**, adrenoceptor; **Gi**, G inhibitory protein.*

6 Aims of thesis

The overall aim of the present thesis was to increase pharmacological knowledge about potential treatment options for cardiovascular dysfunction, caused by accidental hypothermia or occurring during use of therapeutic hypothermia. Treatment of cardiovascular impairment during hypothermia is however complicated, as use of traditional inotropes like β-adrenoceptor agonists is challenging, due to temperature-induced alteration of their effects. Use of inodilators such as PDE3-inhibitors, or simply reducing SVR through drugs that induce peripheral vasodilation, have on the other hand showed promising effects during rewarming (46,115). These pharmacological strategies aim to increase intracellular cAMP in cardiomyocytes and arterial smooth muscle, or to increase intracellular cGMP in arterial smooth muscle. The latter strategy has shown effect down to 15°C with blood pressure reduction by SNP (45). It is however unclear at which temperatures PDE-inhibiting drugs could exert effect on their target molecules and potentially alleviate cardiovascular dysfunction through intracellular increase of cAMP or cGMP. Accordingly, the main research questions discussed in this thesis are:

- How does graded hypothermia affect the capacity of PDE3 and ABCC4 to eliminate cAMP and what effect does low temperatures have on intracellular levels of cAMP in untreated cardiomyocytes?
- Are known PDE3-inhibiting drugs able to reduce cAMP-elimination during graded hypothermia, thereby preserving their pharmacological effect at low temperatures?
- Is the capacity of PDE5 and ABCC5 to reduce intracellular cGMP altered by graded hypothermia and are known PDE5-inhibitors able to reduce such cGMP-elimination at low temperatures?

To answer these questions, we performed in vitro experiments on established models for assessing function of phosphodiesterase enzymes, transport proteins and intracellular cAMP in cultured cardiomyocytes.

6.1 Paper I

The aim of this paper was to explore whether differential hypothermia could have significant effects on the cellular elimination of cAMP and cGMP. The primary aim was to investigate the elimination rate carried out by PDE3 and PDE5, as well as ABCC4 and ABCC5 at six different temperatures, ranging from 37° - 20°C. The secondary aim was to determine baseline levels of cAMP in embryonic H9c2-cells at 37 – 30 – 20°C.

6.2 Paper II

The second paper aimed to examine the pharmacodynamic properties of PDE3-inhibitors milrinone, amrinone, and levosimendan, in hypothermic settings (37° - 20°C). First, we wanted to examine the degree of intracellular access of each study drug. Secondly, we wanted to assess the effects of each drug on PDE3, PDE5, ABCC4, and ABCC5 function, in the same temperature range. Our goal was to provide dose-response curves for each drug and temperature, to illustrate the temperature-dependent activity and determine IC_{50} and K_i values.

6.3 Paper III

The aim was to determine the pharmacodynamic properties of PDE5-inhibitors sildenafil and vardenafil in an identical in vitro setting as in paper II. Similarly, we wanted to assess intracellular access, and determine inhibition curves for both drugs. Hence, we aimed to provide IC_{50} and K_i values for both sildenafil and vardenafil at all included temperatures.

7 Methodological description and considerations

7.1 Ethical considerations

Cardiomyocyte cell culture: A clonal H9c2 cardiac muscle cell line, derived from embryonic rat heart tissue, was used to determine the effect of hypothermia on intracellular cAMP. Use of this clonal cell line enabled us to avoid using research animals for this experiment.

Erythrocytes: Blodbanken (Department of Immunohematology and Transfusion Medicine, University Hospital of North Norway) provided blood for preparation of inside-out vesicles (IOV) and estimation of intracellular access. The blood was randomly selected, pre-screened and only provided if donors were healthy. The experiments were performed according to local legislation and institutional requirements included in our agreement with Blodbanken. The participants provided their written informed consent to contribute before sampling at Blodbanken, and we only received anonymized samples for the experiments. The regional ethical committee found that ethical review and approval was not required for these experiments.

7.2 Pharmaceuticals (II, III)

Five different study drugs, all known phosphodiesterase inhibitors, were included in our project. Seven different concentrations were used for all medications, ranging from 1.00E-09 to 1.00E-03 M (1.00 nM to 1.00 mM) in the final solutions, to test their potency at inhibiting cellular efflux of cyclic nucleotides (7.5.3) and enzyme activity (7.5.4.). For estimating their intracellular access, and thus their ability to reach their primary target (phosphodiesterase enzymes), individual dosages were used based on their varying therapeutic plasma

concentrations in normothermia, which are disclosed in the experimental protocol (7.5.2). The whole therapeutic concentration range of included drugs is covered in the present experiments.

7.2.1 PDE3 inhibitors

Milrinone (United States Pharmacopeia (USP) Reference Standard, Rockville, United States) is usually administered with an initial bolus of 50 µg/kg over 10 minutes, followed by continuous infusion, typically 0.50 µg/kg/min (0.375 - 0.75 µg/kg/min) in patients with congestive heart failure. Therapeutic range for steady-state plasma concentrations is 100 – 300 ng/mL during therapy, although higher concentrations have been measured with conventional dosing (74,128–130). These concentrations correspond to ~ 0.47 – 1.42 µM.

Amrinone (Sigma-Aldrich, Steinheim, Germany) therapy is also initiated with a bolus of 0.75 mg/kg given over 2-3 minutes, followed by continuous infusion, 5 – 10 µg/kg/min (131). The main indication is congestive heart failure. The therapeutic dosage range is 0.5 – 7.0 µg/mL, corresponding to ~ 2.67 – 37.39 µM (75,132,133).

Levosimendan (Sigma-Aldrich, Steinheim, Germany) is usually given to patients as a loading dose of 6 – 12 µg/kg over 10 minutes, followed by 0.05 – 0.2 µg/kg/min as a continuous infusion. In healthy volunteers, steady state serum concentrations ranged from 14.9 to 62.6 ng/mL, depending on infusion rate and length (134). In decompensated heart failure patients (left ventricular ejection fraction 30%), the mean dose of levosimendan, 0.26 µg/kg/min after 6 hours of treatment, yielded approximately 120 ng/mL in plasma (135). In molar concentrations, these values correspond to ~ 53.2 nM (14.9 ng/mL) – 428.1 nM (120 ng/mL).

7.2.2 PDE5 inhibitors

Sildenafil (Sigma-Aldrich, Schnelldorf, Germany) is administered intravenously as a bolus of 10 mg, three times daily, for pulmonal hypertension. A study compared the pharmacokinetics of iv administered (25 mg) versus orally ingested (50 mg) sildenafil in healthy participants (136). These doses were probably selected based on sildenafil's bioavailability of 41% after oral intake (137). Mean C_{max} in plasma was 560 ng/mL after intravenous administration and 610 ng/mL after oral intake (136). However, in patients with pulmonary hypertension, who switched from oral sildenafil treatment (20 mg times 3) to a combination treatment (10 mg iv + 20 mg times 2 orally), C_{max} was 213.3 ng/mL - comparable to the steady state levels with oral medication (138). These plasma concentrations correlate to 0.45 (213.3 ng/mL) – 1.29 (610 ng/mL) µM sildenafil.

Vardenafil (Bayer Pharma AG, Wuppertal, Germany) is taken orally. The recommended dose for adults is 10 mg prior to sexual activity (5-20 mg), leading to a maximal concentration of 9.05 µg/L (10 mg) and 20.9 µg/L (20 mg) within an hour (127,139). In patients with primary and secondary pulmonary hypertension, the mean plasma concentration was merely 5.07 µg/L after an hour despite having the majority intake 20 mg (140). Thus, the concentrations in plasma correspond to ~ 10.38 µM (5.07 µg/L) – 42.77 µM (20.9 µg/L).

7.3 Temperature

Six different temperatures were chosen to represent the full spectrum of hypothermia severity, including normothermia, for all transport and phosphodiesterase enzyme experiments; 37 – 34 – 32 – 28 – 24 – 20°C. Temperature induction and maintenance was conducted by using a Grant Optima T100 heated circulating bath (Grant Instruments LTD., Shepreth England). For

cAMP quantification in the H9c2 clonal cell model, cells were incubated for either 3, 15, or 30 min at 37 – 30 – 20°C. This was performed using a Labwit Snake 90 incubator (Labwit Scientific).

7.4 Cell Preparation

7.4.1 Cardiomyocyte Cell Culture

The first experiment included the clonal cardiomyocyte cell line, H9c2, derived from embryonic BDIX rat heart tissue, provided to us by the Cardiovascular Research group at IMB, UiT. The cells were phenotypically not contractile and not subjugated to electrical stimulation in this project (141).

The H9c2 cells were preserved in Dulbecco's Modified Eagle's Medium with 10% Fetal Bovine Serum, and so-called culture medium; a combination of 1% penicillin-streptomycin and 5% CO_2, at 37°C. The cells were kept in storage in cryotubes at -196°C and was subsequently defrosted by rapid warming in a water bath (37°C) and careful washing with Dimethyl sulfoxide to secure viability. 10 mL of the culture medium was added before centrifugation (5 minutes at 108 g) in a MSE Minor Centrifuge. Supernatant was discarded and additional 15 mL of the culture medium was added. The resulting suspension was consequently transferred to 75 cm^3 culture flasks for incubation.

The cell confluency was deemed sufficient at 70-80% after a few days and the cells were transferred to other 175 cm^3 culture flasks. This cell passaging was carried out by initial aspiration of the culture medium, followed by thorough washing with 10 mL preheated phosphate-buffered saline. 1.5 mL preheated 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) solution was added in each flask before incubation with 5% CO_2 (3-5 minutes, at

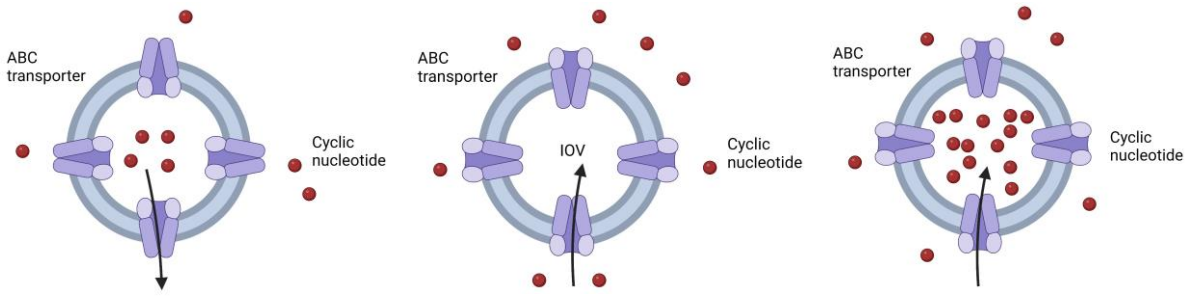
37°C) to promote detachment of the cells. Successful cell detachment was confirmed with a light microscope. Additional 8.5 mL culture medium was pipetted in each flask to end the trypsinization process and supply the cells with nutrients and pH buffering. 1-2.5 mL of the cell suspension was ultimately transferred to several other 175 cm^3 sterile flasks, along with extra culture medium (up to 35 mL). During this cell culturing process, cell density and morphology was evaluated with a light microscope three or four times a week. The culture medium was also regularly replaced to ensure sufficient nutrient levels and stable pH environment. When the cell confluency reached 70-80%, the cell passaging was performed – typically two or three passages a week. The range of passage numbers used in the experiments was 9 to 18. The experiment protocol required three or four 175 cm^3 culture flasks with 70-80% confluency.

Prior to the commencement of the hypothermia experiment, the cells were aspirated, washed and trypsinated, as described for the cell passaging method. The entire cell culture was harvested from the flasks to a single centrifuge tube (50 mL), in which it was resuspended. Cell count and viability measure was performed with a Countess Automated Cell Counter after adding 20 µL Trypan blue (0.4%) in a 1:1 ratio. This count was done in triplicate and the resulting cell concentration was the average of three measurements. To obtain a predetermined cell concentration of 2.5×10^5-3.5×10^5 cells/well, the cell suspension was diluted with culture medium and subsequently distributed to 13 Falcon 6-well Clear Multiwell Plates with 2 mL cell culture in each well. The plates were incubated at 37°C with 5% CO_2 for 48 hours before performing of the experimental protocol.

7.4.2 Erythrocytes

IOV prepared from erythrocyte membrane is a well-established model for the assessment of intracellular access of pharmaceuticals and cellular removal of cyclic nucleotides through ABC-transporters, at UiT The Arctic University of Norway (142–144). Using IOVs allowed us to study a group of transport proteins that are involved in cellular elimination of cAMP and cGMP from cardiomyocytes and vascular myocytes and that could be drug-targets for cardiovascular treatment during hypothermia (145–147). Amongst these transport proteins are ABCC1, ABCC4, and ABCC5 that have been characterized in the erythrocyte membrane, making IOVs a suitable model (148,149).

We received freshly collected anonymized and mixed EDTA blood from healthy donors (n = 35) provided by Blodbanken (Department of Immunohematology and Transfusion Medicine, University Hospital of North Norway). These batches were mixed and used to prepare IOVs. These are vesicles that are generated by mechanically disrupting the cellular membrane and re-attaching the fragments with the cytoplasmic side on the outside. The resulting IOVs contain intact ABC transporters in the cell membrane but are faced in the opposite direction. Thus, these vesicles enable us to control the intracellular environment (surrounding medium) and collect the cyclic nucleotides within. The method for creating IOVs was developed by Steck et al. and is a well-established model in our lab (150).



Figur 1. Inside-out vesicle (IOV). The erythrocyte is converted into an IOV, with the cytoplasmic side facing outwards.

The following procedures were conducted in a cold environment (0-4°C). The first step was to separate the erythrocytes from plasma by centrifugation at 2 300 g for approximately 15 minutes. Plasma and buffy were discarded and the remaining cells were washed three consecutive times with 5 mM Tris-HCl and 113 mM KCl (pH 8.1), and centrifugated once more at 1 000 g. Lysis was induced by adding ten volumes of 5 mM Tris-HCl, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 4 mM KCl (pH 8.1) and washing by repeated centrifugation at 20 000 g for 20 minutes and subsequent resuspension in the same buffer until the ghosts were milky white. We continued with the process of vesiculation by adding 39 volumes of a hypertonic buffer (0.5 µM Tris-HCl, pH 8.2) to one volume of cell suspension and forcing the solution through a 27-gauge syringe needle five times to promote homogenization of the membranes. The resulting IOVs were separated from the right-side vesicles and ghosts by ultracentrifugation (100 000 g) overnight, using a density gradient, ranging from 1.048 g/ml to 1.146 g/ml (Nycodenz, Axis-Shield PoC, Oslo, Norway) in 5 mM Tris, 3 mM KCl, and 0.3 mM EGTA. Consequently, the IOVs were gathered in the uppermost band, which was collected, washed, and resuspended in 1.47 mM KH_2PO_4, 81 mM K_2KPO_4 and 140 mM KCl (pH 7.6). Acetylcholinesterase accessibility test was employed to verify sidedness of the membranes and determine percentage IOV in the solution (151).

7.5 Experimental Protocol

Our project consisted of four independent experiments to determine baseline levels of cAMP, intracellular access of medications, as well as transport- and enzyme-mediated cyclic nucleotide elimination during hypothermia. Thus, four different experimental protocols are described in the following section. Each experiment operated with triplicates for both test and

control solutions and were conducted as three independent procedures (three separate days) for each temperature, leading to a minimum of nine parallels.

7.5.1 Intracellular cAMP quantification in H9c2 cells (I)

The cell culture, distributed in Falcon 6-well Clear Multiwell Plates, were incubated at the selected temperature for either 3, 15, or 30 min. The incubation medium from the cell cultures was aspirated, and 2 mL cooled phosphate-buffered saline was added to each well. After thorough washing, each well was aspirated, and the samples were later handled on ice to prevent further intracellular reactions. We added 25 µL cooled internal standard (25 nM cAMP, Sigma-Aldrich, St. Louis, MO, USA) and 50 µL cooled Milli-Q water to each sample, along with 200 µL cooled perchloric acid (8%) to induce deproteinization. Remaining cells on the well surface were detached by using a cell scraper and confirmed with the light microscope. The incubation solutions were pipetted to Eppendorf tubes, vortexed and further lysed with a probe sonicator (Sonics Vibra-Cell VC130 Ultrasonic Processor, amplitude of 20 µm for 5 seconds). Additionally, 200 µL ammonium bicarbonate (2 M) was instilled in each sample for neutralization and cooled down to -70°C. On the consecutive day, the solutions were defrosted in order to be vortexed and centrifuged in a cooled storage room (4°C) at 13 400 g for 10 minutes. 200 µL of the resulting supernatant was pipetted from each sample to a 96-positions target plate and stored at -70°C until cAMP quantification was performed.

Protein concentration was calculated for our samples, by using cells taken directly from storage at 37°C in 5% CO_2. Thus, they were not exposed to neither internal standard, perchloric acid, nor ammonium bicarbonate. Instead, 275 µL Milli-Q water was added (corresponding to the same volume of 25 µL internal standard, 50 µL Milli-Q water and 200 µL perchloric acid) after removing the cell medium. The cells went through scraping,

vortexing, and sonification in a similar manner as to the test samples. 200 µL Milli-Q water was transferred to each well, as a substitute for the missing volume of ammonium bicarbonate. The samples were stored at -70°C until protein quantification the day after.

Five serial dilutions of cAMP were prepared to create a standard curve for subsequent analysis: 40 – 200 – 1000 – 5000 – 20 000 pM (Sigma-Aldrich, St. Louis, MO, USA).

7.5.2 Intracellular Access of Pharmaceuticals (II, III)

During normothermic conditions, all five medications have their primary target molecule (phosphodiesterase 3 or 5) located intracellularly. An important aspect of assessing the pharmacological effects in cold temperature is to determine their ability to reach their site of action in the cells at different temperatures.

Anonymized and mixed EDTA blood from healthy donors (n=18) was received from Blodbanken UNN. It was initially washed with Krebs-Ringer-Phosphate-Buffer containing glucose (KRPB/G, pH 7.4) and centrifugated for 10 minutes at 1000 g, three consecutive times. Plasma and buffy coat, containing white blood cells and platelets, were removed and KRPB/G was added until the ratio 1:2.5 (blood:KRPB/G) was reached. Hematocrit (Hct) of the cell solution was measured and titrated with additional buffer in order to reach 0.44, and 0.40 in the final incubation solution.

500 µL of the cell solution and 50 µL of the designated drug or Milli-Q water (negative control) were mixed in a test tube and incubated for 30 minutes at the appointed temperature. To end the reactions, the tube was placed on ice and 4 mL of ice cold KRFB/G was added. Washing with KRFB/G and centrifugation at 600 g for 5 minutes was performed three times in total. 50 µL of the resulting erythrocyte suspension was transferred to an Eppendorf tube,

along with 50 µL internal standard; 500 nm IS-Milrinone-d3 (TLC Pharmaceutical Standards Ltd.), 250 nM IS-Sildenafil-d3, or 500 nM IS-Vardenafil-d5 (Toronto Research Chemicals, ON, Canada). Due to lack of corresponding internal standards for amrinone and levosimendan, 250 nM IS-Pentoxyfylline-d4 (Alsachim, Illkirch Graffenstaden, France) was used instead for amrinone and the combination of d4-pentoxyfylline and IS-Sildenafil-d3 was utilized for determining levosimendan concentrations.

All test samples were added 200 mL 0.1 M $ZnSO_4$ to induce cell lysis and were subsequently centrifugated. 30 µL of the solution was used for protein measurement and the rest was mixed with 500 µL acetonitrile before being centrifugated (2 min, 13 400 g). 100 µL were collected from each tube for MS analysis.

7.5.3 Cellular Efflux of Cyclic Nucleotides (I, II, III)

Pre-prepared IOV suspensions were used for this part of the experiment. To assess cellular efflux through the ABC-transporters ABCC4 and ABCC5 during hypothermia, IOVs were incubated with either 20 µM (^{3}H)-cAMP or 2µM (^{3}H)-cGMP (Perkin Elmer, Boston, MA, United States) at the designated temperature, with or without 2 mM ATP (paper I). Seven different concentrations of each medication were added in study II and III.

Initially the following mixture was prepared: 1 M Tris-HCl, 1 M Tris, 200 mM K_2EGTA, 400 mM KCl. A small amount of the solution was withdrawn and used as negative control (Mg-Cl_2, 300 mM), while the majority had Mg-ATP (300 mM) added to it. We transferred 15 µL of the mixtures to a 96-well block (1 mL wells) that was priorly put on ice. To determine the effects of the study drugs on the transporter activity in cold temperatures, 30 µL of the appointed drug and concentrations were simply added to the assigned wells. The whole block

was subsequently centrifugated (74 g in Heraeus Sepatech). Shortly before the incubation, 15 µL of the vesicle suspension was added to each well and mixed thoroughly, as well as centrifugated up to approximately 50-60 g. The final concentrations in the incubation solution (60 µL) were 12 mM Tris-HCL, 8 mM Tris, 1 mM K_2EGTA, 100 mM KCl, 2 mM Mg-ATP/2 mM $MgCl_2$. The block was covered with parafilm and submerged into the water bath.

The assay was terminated after 60 minutes by putting the block on ice and adding ice cold buffer (<4°C), containing 1.47 mM KH_2PO_4, 8.1 mM K_2HPO_4, and 140 mM KCl (pH 7.6). The next step was performed inside a cooling room (~ 4°C): a nitrocellulose membrane (Bio-Rad Laboratories, Feldkirchen, Germany), used for collecting the IOVs through filtration, was soaked with the cold buffer and assembled in a locally designed filtrating stack. 600 µL of the ice-cold buffer was added to all wells in the block and mixed thoroughly. 600 µL of the resulting solutions were transferred from each well to a corresponding well in the filtration construction, and additional ice-cold buffer was added in all wells until the fluid reached the brim. The device had a rack of larger tubes (2 mL, open ended on both sides) assembled on top, that were also filled with ice cold buffer.

The filtration was initiated by activating a suction pump and attaching it to the outlet of the filtrating device. The resulting vacuum helped to propagate all of the solutions through the filtrating construction and through the imbedded membrane, trapping the vesicles on one side. Once all fluid had dissipated, the membrane was extracted carefully and put in a heated air cabinet (37°C) for approximately 2-3 minutes and left in room temperature for some additional time to dry. The resulting accumulation of radioactivity upon the filter was quantified by using Packard TopCount NXT (Packard, Downers Grove, IL, United States) after adding scintillation fluid (MicroScint-O, PerkinElmer, Groningen, Netherlands).

7.5.4 Phosphodiesterase Assay (I, II, III)

Similar to previous protocol, the phosphodiesterase activity was examined during hypothermia without drug exposure, to estimate the temperature-dependent effect of enzyme activity (paper I) and with the range of included concentrations of all drugs (paper II and III). Either 5 µM cAMP or 5 µM cGMP (Sigma-Aldrich, St. Louis, MO, United States) was used as substrate for the PDE3- and PDE5-enzymes, respectively.

Fresh incubation buffer was mixed on the same day of the experiment: 10 mM Tris, 8.2 mM propionic acid, 3 mM magnesium acetate, 1.5 mM EGTA and 0.5 mg/ml BSA, 0.2 mM DTT. The selected pharmaceutical at all included concentrations were previously added in appointed tubes. Hence, each tube contained 10 µL incubation buffer and 30 µL drug suspension (or Milli-Q water for controls). One minute prior to the assay, all tubes were submerged into the water bath and the reaction was initiated by adding 10 µL of either 0.016 units/µL PDE3 (Abcam, Cambridge, United Kingdom) or 0.022 units/µL PDE5 (Sigma-Aldrich, St. Louis, United States)) The reaction was terminated after 30 minutes by adding 150 µL LC-MS grade methanol to the solutions. 25 µL of internal standards cGMP-^{13}C5, cAMP-^{13}C5, AMP-^{13}C5 (Toronto Research Chemicals Inc., Ontario Canada), and GMP-^{15}N5 (Sigma-Aldrich, St. Louis, MO, United States) were added to each sample prior to the mass spectrometry (MS) analysis. The final concentrations were 10 nM/50 nM (cAMP/AMP) and 10 nM/100 nM (cGMP/GMP). Additionally, 500 nM IS-Milrinone-d3 (TLC Pharmaceutical Standards Ltd.), 250 nM IS-Sildenafil-d3, or 500 nM IS-Vardenafil-d5 (Toronto Research Chemicals, ON, Canada) were added as internal standards where applicable.

The final concentrations of PDE3 inhibitors for the calibration curves, used for mass spectrometry (MS) analysis, were 5000 nM, 1000 nM, 100 nM, 10 nM, 1 nM, and 0.1 nM. Likewise, a 5-point calibration curve for the cyclic nucleotides/non-cyclic nucleotides was

prepared with the final concentrations after adding internal standards: 2000/200 nM, 200/20 nM, 20/2 nM, 2/0.2 nM, and 0.2/0.02 nM.

7.6 Mass Spectrometry Analysis

Liquid chromatography tandem mass spectrometry (LC-MS/MS) measured the concentrations of cAMP levels in the H9c2 cells and drug concentrations for the intracellular access assessment. The levels of cAMP/AMP and cGMP/GMP from the PDE assays were also determined. Each measurement had a concomitant calibration curve and internal standard, where possible.

Stock solvents of cyclic nucleotides/non-nucleotides (2.5/0.25 mM), milrinone (2.5 mM), amrinone (2.5 mM), levosimendan (0.1 mM), sildenafil (2.5 mM), and vardenafil (1 mM) were mixed in methanol and MilliQ H_2O (Millipore SAS, Molsheim, France), in ratio 1:1 and subsequently stored at -20°C. These stocks were used for dilution and preparation of the calibration curves for analysis.

The linearity was found to be from 0.2 nM to at least 2000 nM ($r^2 > 0.998$) for cAMP, AMP, and cGMP. For GMP the range went from 2 nM to at least 2000 nM ($r^2 > 0.998$). Concerning PDE-inhibitors, the linearity went from 10 nM to at least 5 000 nM ($r^2 > 0.99$ for PDE3, $r^2 > 0.998$). Lower limit of quantification (LLOQ) was 0.2 nM for cAMP, cGMP, and AMP. LLOQ for GMP was 2 nM and 10 nM for all PDE-inhibitors.

7.7 Bioactivity

IC_{50} and K_i values are often used to quantify the ability of a drug to inhibit enzymatic activity. IC50 expresses the concentration needed to inhibit half of the maximal enzymatic process (figure 1) and the inhibition constant, K_i, is the concentration of a drug needed to occupy 50% of the designated receptors (152).

IC_{50} values were calculated for the drugs' ability to inhibit cyclic nucleotide efflux through ABC-transporters and PDE activity according to Chou (153). K_i was consequently obtained for all substances (154). The resulting data was illustrated by using dose-response (inhibition) curves, both with and without adjustment for normothermic controls.

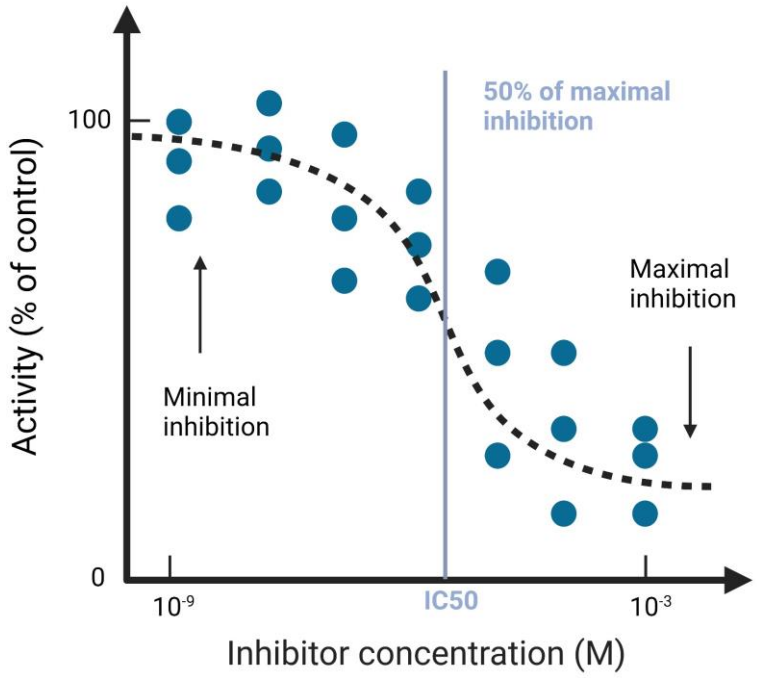


Figure 4. Dose-response curve. Each dot represents a measurement – seven different concentrations and three parallels for each concentration. IC50, half maximal inhibitory concentration, is the concentration where the curve passes the 50% inhibition level.

7.8 Statistical Analysis

The intracellular cAMP concentration in H9c2-cells were corrected for protein concentrations with a Bradford protein assay. Similarly, both the intracellular drug concentrations and the total incubation concentrations in each sample were adjusted for protein concentrations, in order to evaluate the degree of access in percentage. One-way ANOVA with Holm-Sidak multiple comparisons *post hoc* test was performed to compare the IC_{50} and K_i values, and the intracellular drug concentrations at each temperature. When the data was not normally distributed, ANOVA on ranks with Dunn *post hoc* test was used instead.

In paper III, IC_{25}-IC_{75} were estimated using with polynomial cubic regression, based on the inhibition curve of each experiment. Regression analysis was performed to evaluate whether there existed a linear relationship between IC_{50} values and temperature for inhibition of both enzymes and ABC transporters by sildenafil and vardenafil. The Pearson correlation coefficient (r) was calculated for every regression analysis to evaluate how well the calculated lines fitted with the observations.

The results were presented as means ± standard error of mean (SEM). We considered *p*-values < 0.05 to be significant results. Statistical analysis took place in SigmaPlot 14.0 (Systat Software, San Jose, CA, United States).

8 Summary of results

8.1 Paper I

In this in vitro study, we wanted to elucidate the isolated effects of hypothermia on key proteins involved in cyclic nucleotide elimination, and also determine baseline levels of cAMP in cardiac myocytes during differentiated hypothermia.

8.1.1 Intracellular cAMP quantification in H9c2 cells

Intact H9c2 cell colonies were incubated at 37°C, 30°C, and 20°C, for either 3, 15, or 30 minutes. Subsequent preparation of incubation solution and MS measurement revealed significantly increased cAMP at 30°C after 3 and 15 minutes, while 30 minutes of incubation did not generate the same elevation. Lowering temperature to 20°C did not lead to any significant changes, regardless of exposure time.

8.1.2 Cellular efflux of cyclic nucleotides

After incubating IOVs with either cAMP or cGMP for 60 minutes, it was evident that cyclic nucleotide removal through transporter proteins was active at all temperatures (37°C - 20°C). At 28°C, 24°C, and 20°C the levels of cAMP-transport were significantly reduced. cGMP-efflux was significantly lower at 24°C and 20°C compared to 37°C.

8.1.3 Enzymatic breakdown of cyclic nucleotides

cAMP was incubated with PDE3 and cGMP with PDE5 for 30 minutes at the designated temperature. The amount of the resulting degradation products, AMP and GMP, were considered as surrogate parameters for PDE activity.

AMP concentrations were significantly reduced at all lower temperatures, except for at 32°C. Evidently, the biggest drop was seen between 37°C and 34°C, and the PDE3 activity was more than halved at 28°C. GMP levels were significantly lower at 28°C, 24°C, and 20°C compared to 37°C. Similar to PDE3-activity, PDE5 activity was halved at 28°C.

8.2 Paper II

In this paper, we investigated the effects of PDE3-inhibitors milrinone, amrinone, and levosimendan on PDEs and ABC-transporters in hypothermia (37°C - 20°C). In three separate essays, each individual study drug was assessed regarding its ability to reach its cytosolic target and its ability to inhibit PDE activity and ABC-transporter function.

8.2.1 Intracellular access

The study drugs were incubated separately along with intact erythrocytes in an incubation solution for 30 minutes at six different temperatures. Decreasing the temperature gradually from 37°C to 20°C did not lead to significant changes in the drugs' ability to reach their intracellular site of action.

8.2.2 Cellular efflux of cyclic nucleotides

Similar to experiment 8.1.2 in the previous paper, IOVs were incubated with either radioactive cAMP or cGMP and the appointed study drug for 60 minutes. The radioactivity signals of the cyclic nucleotides inside of the IOVs were measured afterwards and were indicative of the ABC-transporter function during simultaneous hypothermia and pharmaceutical influence.

Milrinone

Lowering the temperature did not significantly alter potency of milrinone, but at 24°C and 20°C, none of the studied drugs were able to inhibit cAMP-efflux through ABCC4. Lower concentrations were needed to inhibit ABCC5 at 24°C and 20°C, compared to 37°C.

Amrinone

There were no significant changes in potency at temperatures 34 – 32 – 28°C, compared to 37°C. Amrinone was not able to inhibit cAMP-efflux at 24°C and below. There were no significant changes in ABCC5-inhibition during cooling, although a trend towards higher IC_{50} and K_i mean values were noted at 20°C.

Levosimendan

Levosimendan inhibited ABCC4 at all temperatures down to 28°C. Like the other study drugs, the potency remained unchanged between 37°C and 28°C but levosimendan was not able to inhibit cAMP-efflux at 24°C and 20°C. Inhibition of ABCC5-mediated cGMP-efflux by levosimendan was unchanged by hypothermia.

8.2.3 Enzymatic breakdown of cyclic nucleotides

cAMP was incubated with PDE3 and cGMP with PDE5 for 30 minutes at each temperature, along with one of the three study drugs in separate experiments. The quantity of the resulting degradation products, AMP and GMP, were considered as surrogate parameters for PDE activity.

Milrinone

Compared to 37°C there were no significant changes in IC_{50} and K_i values for inhibiting either PDE3 or PDE5 at lower temperatures, compared to normothermia.

Amrinone

Amrinone was able to inhibit both PDE3 and PDE5 at all temperatures, without any significant changes in potency.

Levosimendan

Comparable to the effects of the other study drugs, IC_{50} and K_i values for inhibiting PDE3 and PDE5 remained unaffected in cooler temperatures.

8.2.4 Drug selectivity

Phosphodiesterase enzymes

To determine if selectivity for target molecules were altered by hypothermia, IC_{50} ratios for the different enzymes and transport proteins were compared. Not surprisingly, higher concentrations of all drugs were needed to inhibit PDE5, compared to PDE3, in normothermia. This effect was enhanced for levosimendan at 32°C - 28°C, with a doubled IC_{50} ratio for PDE5/PDE3 inhibition, before returning to normothermic selectivity at 24°C

and 20°C. Amrinone was the least selective drug at all temperatures, with a more similar IC_{50}-value for all included target molecules.

Cyclic nucleotide efflux

Drug selectivity for cGMP versus cAMP efflux was assessed. Milrinone and amrinone appeared to inhibit cGMP expulsion at lower concentrations than cAMP efflux at 37°C. The IC_{50} ratio for levosimendan indicated inverse selectivity at 37°C compared to amrinone and milrinone but this changed during cooling to 28°C.

cAMP elimination

The IC_{50} ratio for milrinone and levosimendan concerning ABCC4/PDE3 revealed a selectivity towards higher effect on enzymatic elimination at 37°C (M: 19.66, L: 28.76). The ratio nearly doubled at 34°C for milrinone and increased steadily for levosimendan with decreasing temperature. In contrast, amrinone showed preference towards ABCC4-inhibition at all temperatures, reflected in a consistent IC_{50} ratio < 1.

cGMP elimination

There was higher selectivity towards cellular efflux inhibition than PDE5-mediated degradation for all medications at all temperatures, as shown by the persisting IC_{50} ratio < 1.

8.3 Paper III

In the last paper, the effects of PDE5-inhibitors sildenafil and vardenafil on PDEs and ABC-transporters were evaluated in hypothermia (37°C - 20°C). The experiments were identical to those described in paper II, with three separate essays, where both drugs were evaluated individually.

8.3.1 Intracellular access

Sildenafil and vardenafil were incubated separately with intact erythrocytes in an incubation solution at all included temperatures. They were able to reach the cytosol in equal amounts at all temperatures after 30 min incubation.

8.3.2 Cellular efflux of cyclic nucleotides

Sildenafil

When compared to the normothermic control, there were not statistically differences in IC_{50} and K_i values for inhibiting ABCC5 or ABCC4 in lower temperatures. None of the medications were able to inhibit ABCC4 at 24°C and below.

Vardenafil

Lowering the temperature from 37°C did not lead to any statistical changes in IC_{50} and K_i values for inhibiting ABCC5 and ABCC4.

8.3.3 Enzymatic breakdown of cyclic nucleotides

Sildenafil

IC_{50} and K_i values for PDE5-inhibition were significantly increased at both moderate and severe hypothermia, with the exemption at 28°C. Concerning PDE3-inhibition, significantly higher dosages of sildenafil were required to achieve the same degree of inhibition at 20°C as in normothermia.

Vardenafil

IC_{50} and K_i values for vardenafil inhibition of both PDE3 and PDE5 did not differ significantly from the normothermic values, although a trend of increasing values were noted for PDE3-inhibition.

8.3.4 Drug selectivity

Phosphodiesterase enzymes

Calculated ratios between IC_{50} values demonstrated that substantially higher concentrations of sildenafil were needed to inhibit PDE3 than PDE5 at all temperatures, but the ratio was reduced by 50% of the normothermic value at 20°C. Vardenafil was comparably less selective at all temperatures. Different from sildenafil, hypothermia seemed to increase the PDE5-selectivity of vardenafil.

Cyclic nucleotide efflux

IC_{50} ratios for sildenafil and vardenafil were quite similar at 37°C. Lowering the temperature led to moderate increases in selectivity towards inhibiting cGMP-efflux for both medications.

cGMP elimination

In normothermia, cGMP-efflux/PDE5-inhibition ratio showed that cGMP elimination is predominantly through PDE5 hydrolysis for both medications. The ratios decreased for both as the temperature fell, with the biggest impact on sildenafil at all subnormal temperatures.

cAMP elimination

The cAMP-efflux/PDE3-inhibition ratio for both sildenafil and vardenafil were consistently < 1, exhibiting a predilection for reducing cAMP-efflux at all temperatures.

9 General discussion

In general, little is known about pharmacokinetic and pharmacodynamic effects of drugs at temperatures below 37°C. It is widely known that enzymatic processes are temperature-dependent and that low temperatures have considerable impact on all organ systems. Furthermore, hypothermia may influence these pharmacological properties differently depending on the drug, rendering prediction of clinical outcome of treatment challenging during hypothermia and rewarming (155). To this date, no evidence-based pharmacological guidelines for treating cardiovascular complications in accidental hypothermia below 30°C exist (4). Yet, it is common practice to provide pharmacological, cardiovascular support in the clinical setting, regardless of core body temperature.

With this project, our first goal was to explore the isolated effects of differential temperatures on cellular elimination of cAMP and cGMP that are central in cardiovascular regulation and important targets for cardiovascular support both in normothermic and hypothermic patients (82). This provided foundation for investigating the direct impact of hypothermia on the pharmacodynamic effects of five selected PDE-inhibitors at temperatures down to 20°C, chosen for their potential clinical utility in treatment of hypothermic patients.

Our research showed that both moderate and severe hypothermia had a suppressive impact on the cellular elimination of cyclic nucleotides. The activity of both metabolizing enzymes and transporter proteins was reduced. Interestingly, measuring intracellular cAMP concentrations in cultured H9c2 cells, revealed higher levels at 30°C (moderate hypothermia) but not at 20°C (severe hypothermia), compared to normothermia (37°C). These findings suggest that moderate hypothermia has diverging effects on the production and elimination of cAMP.

During cooling, the PDE3 activity declined, starting at moderate hypothermia (34°C), with only one-fourth of the activity remaining at 20°C. Simultaneously, ABCC4 function started

declining in severe hypothermia (28°C) and at 20°C, less than 10% of the normothermic activity was measured. Thus, higher cAMP levels in cultured H9c2 cardiomyocytes at 30°C could be explained by a lower rate of cAMP elimination through degradation and cellular expulsion, while the production rate is less affected. In contrast to at 30°C, cAMP levels in the same cells were similar to normothermic levels at 20°C, which could be explained by both lowered production and elimination of the cyclic nucleotide during severe hypothermia.

9.1 Hypotherma-induced cardiac failure

The cellular pathophysiology of heart failure in normothermia is complex, including both altered regulation of cAMP and cGMP signaling, as well as impaired regulation of $[Ca^{2+}]_i$ (156). Similarly, dysregulated Ca^{2+} hemostasis has been shown to play an important role in the development of HCD and fulminant rewarming shock (25,26,157). In a normal physiological state, $[Ca^{2+}]_i$ is kept low until electrical stimulation and depolarization of the cell take place. This incites entrance of extracellular Ca^{2+} into the cell through L-type calcium channels, which in turn elicits high Ca^{2+} flux from the SR. This increase of Ca^{2+} in the cytosol initiates contraction: specifically, Ca^{2+} binds to Troponin-C which induces conformational changes in the troponin-tropomyosin complex and thereby exposes myosin-binding sites on actin. Ultimately cross-bridges form between actin and myosin which produce sliding movements of the myofilaments and results in muscle contraction (158,159). The response of transient calcium flux seems to be particularly increased and prolonged in mild and moderate hypothermia, resulting in increased inotropy (31,160). One of the mechanisms underlying this phenomenon is altered Ca^{2+} release and reuptake by the SR. The cardiomyocyte is thought to initially be able to adapt to the accumulation of Ca^{2+} during diastole by reducing the rate of release, thereby keeping the net systolic concentration under control, and maintaining the

contractile work. As the temperature drops, it becomes increasingly difficult to maintain homeostasis, despite very prolonged release and reuptake of Ca^{2+}. The ensuing Ca^{2+} overload during both diastole and systole causes impairment in relaxation, as well as a decline in contractility (157,161).

One of the roles of cAMP is to activate protein kinase A (PKA), which in turn raises the calcium current through voltage-dependent L-type Ca^{2+} channels in the sarcolemma and increases the Ca^{2+} release and reuptake conducted by the SR through ryanodine receptors. However, when PKA is activated in an inappropriate manner, heart failure follows as a result of several downstream reactions. In transgenic mice, engineered to express chronic cardiac PKA activation, dilated cardiomyopathy with reduced contractility, arrhythmias, and in some cases sudden death ensued (156,162). PKA also regulates the myofilament function by phosphorylating cTnI and myosin-binding protein C. The phosphorylation of cTnI at Ser23/24 (in the NH_2-terminal extension) is known to reduce the myofilament Ca^{2+} sensitivity after physiological β-adrenergic stimulation and subsequent stimulation of PKA (163). Hypothermia and following rewarming seem to exaggerate this modulation and thereby contribute to HCD (164,165). Thus, the pathophysiology of heart failure in accidental hypothermia comprises both a progressive Ca^{2+} loading in the cells and decreased calcium-sensitivity, both of which are downstream reactions to an inappropriate cAMP-PKA signaling. Hence, although cAMP levels in severe hypothermia were similar to normothermic values in our untreated cardiomyocytes, these levels could be excessive in regard to the physiological state at that temperature. This is clearly illustrated in a rodent model, where intracellular cAMP levels at 15°C were unchanged compared to the baseline normothermic levels. Five minutes of adrenaline-administration at this temperature gave a four-fold increase of cAMP (compared to two-fold in normothermia) but failed to raise SV and CO, as in normothermia. Instead, a detrimental four-fold increase in SVR was observed in combination

with a substantial reduction of CO and SV, indicating a toxic effect of adrenaline during severe hypothermia (84).

9.2 Cold-induced vasoconstriction

It is well-known that peripheral arteries have increased α-adrenergic mediated vasoconstriction at low temperatures. Consequently, SVR has been consistently elevated in animal studies examining moderate and severe hypothermia, as well as in human studies examining cardiovascular responses to cold exposure (166). After rewarming and maintained normothermic core temperature, the vascular resistance fail to return to baseline levels (23,167). This is not the case in larger, deeper vessels. In a study conducted by Chung et al., cooling isolated rat aorta with intact endothelium to 25°C led to vasodilation. Simultaneously, radioimmunoassays revealed significantly increased cAMP and cGMP levels in the vascular smooth muscle cells as direct responses to the cold: consequently, it was evident that α_1-adrenoceptor-mediated contraction was attenuated at this temperature as the phenylephrine dose-response curve shifted right- and downward (168). These findings suggest that the endothelium-dependent vasorelaxation through NO – cGMP pathway, amongst other mechanisms, is intact during severe hypothermia and that decreased elimination rate of cGMP, but unaffected synthesis, could be a plausible explanation for aortic vasodilation. Several other studies have also concluded that hypothermia induces vasodilation, rather than vasoconstriction, in deeper vessels (169,170). Canbolat and Atalik demonstrated reduced sensitivity to calcium channel blockers in calf cardiac veins at 28°C in the presence of N^G -nitro-L-arginine methyl ester, which is a known nitric oxide synthase (NOS) inhibitor, compared to the isolated effects of the blockers (171). Thus, it seems that lower temperatures do not extinguish NO-dependent vasorelaxation.

Several different mechanisms for cold-induced vasoconstriction have been described. Reflex cutaneous vasoconstriction is mediated through sympathetic nerves and reacts immediately to cold exposure (172). The vasoconstrictor nerves release predominantly noradrenaline as their neurotransmitter, which act primarily through α_1-adrenergic receptors and α_2-adrenergic receptors to some extent (173,174). However, additional mechanisms with other vasoconstrictor cotransmitters have been identified that make up to 30% of the vasoconstrictor response (172,175). Stimulating α_1-adrenergic receptors leads to vasoconstriction through the IP_3 signal transduction pathway, while the activation of α_2-adrenergic receptors decreases cAMP levels (176,177). Local skin cooling initiates vasoconstriction by upregulating postsynaptic α_{2c}-adrenergic receptors, and thereby amplifying the inhibitory effect on cAMP production, as well as inhibiting the NO system, hence diminishing the production of both cAMP and cGMP (178). Thus, different intracellular downstream mechanisms interact to induce smooth muscle cell contraction after skin cooling.

Also reduced core temperature affect intracellular cyclic nucleotide levels. We demonstrated lower rates of cGMP elimination in severe hypothermia (28°C and lower) and slower cAMP removal starting in moderate hypothermia (34°C and lower) in our project. It is evident that although hypothermia decreases cyclic nucleotide degradation, their physiological effects seen in normothermia are counteracted by other processes. In the present project, our findings indicate that cardiomyocyte cAMP-production is reduced by severe hypothermia but not by moderate hypothermia. This correlated with the known positive inotropic effect of mild to moderate hypothermia on the heart (30,31). In the present work, we did not investigate the intracellular levels of cyclic nucleotides in vascular smooth muscle cells, but it could be speculated that the increased SVR previously observed during severe hypothermia in experimental animal and patients, could be caused by lower intracellular levels of cyclic

nucleotides in arterial smooth muscle cells compared to normothermia. This could, however, also be a consequence of other hypothermia-induced processes, such as reduced elimination and prolonged half-life of vasoactive substances, such as adrenaline and noradrenaline.

9.3 PDE inhibitors in hypothermia

In the recent years, additional information has contributed to further the understanding of the potential use of PDE inhibitors in accidental hypothermia. The majority of available data concerns the use of PDE3 inhibitors milrinone and levosimendan and is extracted from both animal studies and clinical experience from therapeutic hypothermia patients. Our basic research illustrate intact function of both PDE3 and PDE5 inhibitors on target molecules in temperatures down to 20°C, with some minor alterations in drug potency (179,180). Drug selectivity was estimated for both groups: lower temperature reinforced the selectivity for PDE3 inhibitors towards enzyme inhibition (PDE3), rather than impeding ABCC4 function, while it was the opposite trend in regard to PDE5 inhibitors and target molecules (PDE5 vs ABCC5). Nevertheless, all drugs continued to exhibit considerable selectivity towards enzyme inhibition (PDE3 and PDE5) at all temperatures, except for amrinone.

These results harmonize with some of the previous research that have demonstrated persisting clinical effects of both milrinone and levosimendan in moderate hypothermia (32-28°C), albeit sometimes in an exaggerated manner when administered conventionally (118,123). Inappropriate blood pressure reduction, which sometimes necessitates the use of vasopressor to maintain acceptable hemodynamic parameters, is perhaps to be expected as the target proteins are already affected by hypothermia alone. Adding functionally active pharmaceuticals in this setting further propagates the inhibition and give toxic effects at doses considered therapeutic in normothermia.

In animal studies, both increased inotropy and vasodilation were however evident without adverse effects on the arterial pressure. These positive outcomes were manifested primarily after rewarming and not during hypothermia. Although the positive hemodynamic effects were difficult to detect before rewarming reached temperatures close to normothermia, treated animals did not experience HCD like their controls (31,46,120). Evidently, the present thesis and previous studies show that milrinone is able to increase cAMP levels and exert positive cardiovascular effects during hypothermia and rewarming (114,115).

9.4 ABC transporters in hypothermia

ABCC4 and ABCC5 are part of the ABC transporter family and are defined as primary active transporters. By cleaving ATP and using the released energy, these transporters are able to move substances from areas of low concentration to others of high concentration (68). They have become increasingly important as pharmacological targets for treatment of various diseases, due to their role in developing multidrug resistance in cancer chemotherapy and infectious diseases (181). A previous work investigated the effects of moderate hypothermia on ABCB1, which transports exogenous substances out of various cells, to determine pharmacokinetic changes. Not surprisingly, already at 32°C, ABCB1-mediated transport of digoxin, a known substrate for the transporter, decreased significantly and continued to decline till 4°C. In contrast, the net transport ratio of quinidine, another ABCB1 substrate, was not reduced until at 4°C. Besides this discrepancy concerning transportation of different substances through the same protein, this study also demonstrated intact passive diffusion through the cell membrane and paracellular transportation at 25°C and higher temperatures (182). To our knowledge, no other studies investigate the function of ABC transporters in hypothermia beside the above-mentioned article and the papers included in this thesis. We

concluded that ABCC4 function was significantly reduced at 28°C and below, and ABCC5 at 24°C – findings which could cause toxic overload of both endogenous and exogenous substances when core temperature falls below these levels. Together with reduced PDE3-function, such hypothermia-mediated ABCC4 dysfunction could also explain the massive cAMP increase observed in rat cardiomyocytes after exposure to adrenaline in vivo during spontaneous circulation at 15°C (84).

9.5 Inodilation versus vasodilation

As HCD is characterized by contractile failure and elevated SVR, attempts have been made to counteract the pathophysiology and find optimal treatment strategies for cardiovascular support. The objective is to restore organ perfusion and optimize the microvascular circulation, preferably without any adverse effects. It has been shown that treatment with either milrinone or levosimendan, in absence of other cardiovascular drugs, mitigates the hypothermic effects on the circulation in cold rats by supporting cardiac function directly and indirectly by reducing SVR (46,120). In contrast, SNP, which has also demonstrated positive effects on cardiac activity through its assumed solitary influence on the peripheral vasculature, is not able to completely restore the function as shown with levosimendan and milrinone (45). Håheim et al. wanted to go further by comparing these two different strategies and their effects on organ blood flow by administering either SNP or levosimendan during rewarming from severe hypothermia. A similar rat model and hypothermia/rewarming protocol were used as in the previous milrinone and levosimendan studies. As previously reported, the levosimendan group exhibited superior cardiovascular support compared to nitroprusside during and after rewarming. Yet, the drugs increased the brain blood flow equally after rewarming. Additionally, only SNP was able to elevate the myocardial blood

flow, indicating that vasodilation might have a stronger impact on organ perfusion and ultimately the microcirculation than the inodilators (113).

9.6 Future implications and other drugs candidates

This project was conducted in vitro to investigate the direct effects of included pharmaceuticals on their target molecules during differentiated hypothermia. Consequently, other potentially interfering reactions in the cells were omitted, as well as pharmacokinetic factors. Valuable knowledge concerning pharmacological properties of PDE inhibitors in hypothermia has been obtained but as with all basic research, one must be careful when translating experimental scientific results into clinical practices.

Treatment of hypothermic patients in the prehospital and in-hospital setting depends on several factors: the clinical status of the patient, the stage of hypothermia, and the resources available. Extracorporeal rewarming, more specifically veno-arterial ECMO is recommended for all patients with hypothermic cardiac arrests or hemodynamic instability during hypothermia (2,4). This modality is quite advantageous since it allows for gradual and controlled rewarming rates and supports both the respiratory and the cardiovascular systems during the critical phases. The use of ECMO has made it possible to treat patients with core temperatures as low as 11.8°C. In 2020, a 27-month-old toddler was successfully treated with prolonged ECMO-aided rewarming after being found outside without any signs of life. The initial rectal temperature was 12.6°C, which dropped to 11.8°C after 10 minutes of reperfusion. The boy was discharged after 64 days with only peripheral paresthesia and some limitation to precise movements of the extremities (183). Thus, rewarming with ECMO is associated with higher survival rates compared to CPB and also permits improved

neurological outcomes (61,184). It is therefore not surprising that the use of ECLS has become more widespread and that it is considered the golden standard for treating HCD.

One of the apparent limitations is the availability of such modalities as not all medical centers can provide ECLS and those that do, have a limited number of machines. The procedure is expensive and requires specialized training to manage as well as extra supporting personnel. Although, the use of ECLS has advanced into prehospital settings, it is still a scarce resource and moving hypothermic patients to the nearest ECLS center for in-hospital treatment often gives prolonged transfer times. Thus, the main challenges with current treatment algorithms are both logistical and economical. Improved pharmacological therapy could be beneficial, as it would be easier to provide to all patients. Such treatment could easily start in the pre-hospital setting, also in low-resource environments.

There is a need for deeper understanding of biochemical processes during hypothermia to improve pharmacological treatment of this patient group. This would lay for an evidence-based pharmacological approach to alleviate HCD and other hypothermia-induced complications, like arrhythmias and rescue collapse. Research on PDE inhibitors, both in vitro and in vivo, has shown promising results where catecholamines largely have failed (82,115,185). It raises the question of whether novel drugs, targeting the same enzymes and/or transporter molecules, could provide better hemodynamic support with a superior safety profile. ITI-214 (PDE1 inhibitor) and PDE9-I (PDE9 inhibitor) are both potent inodilators that are being investigated as potential drugs for treating heart failure in normothermia (156,186,187). It is plausible that these medications also could have positive cardiovascular effects during hypothermia and rewarming.

ABCC4 has also been investigated as a potential target for treatment of cardiovascular diseases, as the transport protein has been found both in human coronary artery smooth

muscle cells and cardiomyocytes (188). Furthermore, several different compounds have been constructed by virtual ligand screening as potential ABCC5 inhibitors – derivatives of PDE5 inhibitors sildenafil and vardenafil, some of which are more potent than the original drugs (189,190). Hence, there is a considerable potential for developing commercial pharmaceuticals that target the cyclic nucleotide regulation through ABC transporter activity. Future research will unfold the full capacity of this drug group.

Novel pharmacological strategies have a high potential for improving treatment of accidental hypothermia, as this is a serious condition with high mortality rates. Affected patients are often unconscious and undergoing CPR upon admission. As such, there is little room for errors if the resuscitation attempts should succeed. Evidence-based guidelines for pharmacological support are however lacking, as there are no randomized clinical studies investigating potential life-saving treatments. Consequently, most research concerning accidental hypothermia is based on animal models and clinical studies involving elective surgeries and therapeutic hypothermia. Occasional successful case reports have contributed to the current knowledge that we possess today.

As the investigated patient populations have been heterogenous and the study designs quite diverse, outcomes have fluctuated as well. Furthermore, the clinical context with differing patient demographics implies wide variations in pharmacokinetics and pharmacodynamics of candidate-drugs during the rewarming phase. As shown in the present thesis, one of the most important implications of hypothermia is to modify dosages in order to reduce the risk of complications. It is therefore vital to further investigate temperature-dependent pharmacological effects of PDE-inhibitors and other potential resuscitation drugs at temperatures <37°C.

10 Conclusions

The main aim of this thesis was to increase pharmacological knowledge about potential treatment options for cardiovascular dysfunction in hypothermic patients. We wanted to investigate at which temperatures PDE-inhibiting drugs could exert effect on their target molecules and potentially alleviate cardiovascular dysfunction through intracellular increase of cAMP or cGMP. Our main conclusions based on the experimental results are:

- Both ABC transporters and PDEs exhibit temperature-dependency in the elimination of intracellular cyclic nucleotides. Significant activity reduction is already apparent at 34°C for PDE3. Intracellular cAMP levels are increased during moderate hypothermia, likely due to preserved production and reduced elimination.

- PDE3-inhibitors milrinone, amrinone, and levosimendan are all functionally active in a hypothermia setting. Lowering the temperature does not significantly alter the drug potency for inhibiting PDEs. None of the medications were able to impede ABCC4 below 28°C.

- PDE5-inhibitors sildenafil and vardenafil are able to inhibit all tested target molecules at all temperatures, except for ABCC4, below 28°C. Vardenafil remains equally potent in moderate and severe hypothermia, while higher concentrations of sildenafil are needed to impede both PDE3 at 32°C and 20°C, and PDE5 function at 20°C.

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Moderate but not severe hypothermia increases intracellular cyclic AMP through preserved production and reduced elimination

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ABSTRACT

Rewarming from accidental hypothermia could be complicated by acute cardiac dysfunction but providing supportive pharmacotherapy at low core temperatures is challenging. Several pharmacological strategies aim to improve cardiovascular function by increasing cAMP in cardiomyocytes as well as cAMP and cGMP levels in vascular smooth muscle, but it is not clear what effects temperature has on cellular elimination of cAMP and cGMP. We therefore studied the effects of differential temperatures from normothermia to deep hypothermia (37 °C–20 °C) on cAMP levels in embryonic H9c2 cardiac cells and elimination of cAMP and cGMP by PDE-enzymes and ABC-transporter proteins. Our experiments showed significant elevation of intracellular cAMP in H9c2-cells at 30 °C but not 20 °C. Elimination of both cAMP and cGMP through ABC transport-proteins and PDE-enzymes showed a temperature dependent reduction. Accordingly, the increased cardiomyocyte cAMP-levels during moderate hypothermia appears an effect of preserved production and reduced elimination at 30 °C. This correlates with earlier in vivo findings of a positive inotropic effect of moderate hypothermia.

1. Introduction

Core temperature in humans is tightly regulated through central and peripheral thermoregulatory mechanisms, providing minimal variation during normal conditions [27]. However, in situations where cold exposure exceeds the body's physiological adaptation and resources, hypothermia ensues [17]. Accidental hypothermia is defined as an involuntary drop in core temperature below 35 °C and there are several staging systems available for grading the severity of this condition based on measured core temperature [22,25]. Traditionally, clinical manifestations have been used to estimate the severity in situations where core temperature measurement was not available [12]. Initially, during mild hypothermia (35°C–32 °C), symptoms like shivering, tachycardia and raised blood pressure, are present. Moderate hypothermia (32°C–28 °C) induces progressive bradycardia, risk for arrhythmias and increased systemic vascular resistance (SVR) with resulting decrease in cardiac output (CO) [10,23,27]. In severe hypothermia (<28 °C), the risk of asystole increases substantially [9,13]. Once transferred into an intensive care unit, rewarming patients with spontaneous circulation is associated with several complications such as hypothermia-induced cardiac dysfunction. If left untreated, there is a high risk that cardiac output (CO) fails to meet the body's metabolic demand or that cardiac arrest ensues [2,9,35,36], with a fatal outcome for the patient.

The underlying mechanisms for the pathophysiological changes observed during cooling and rewarming are not yet fully understood. In healthy and normothermic individuals, cardiovascular function is regulated through changes in intracellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [11,32]. cAMP is important for regulation of cardiomyocyte function and myocardial contractility, while both cAMP and cGMP are involved in regulation of vascular smooth muscle dilation. Consequently, several pharmacological approaches targeting both nucleotides have been

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developed to treat cardiovascular disorders [39]. Regulation of cGMP and cAMP levels could be achieved through regulating production, mediated by stimulation or blockade of receptors in the cell membrane. Another option is to alter elimination, either through degradation by various phosphodiesterase enzymes (PDE) or cellular extrusion, mediated by membrane-bound ABC-transporters [1,31].

Phosphodiesterase 3A (PDE3A) is mainly responsible for degrading cAMP in cardiomyocytes whereas PDE5A, which predominantly is found in vascular smooth muscle cells, metabolizes cGMP [26,33]. cAMP is actively exported out of the cell through transporter proteins belonging to the ATP-binding cassette subfamily-C 4 (ABCC4) and cGMP through ABCC5 [16,37]. It is widely known that enzymatic processes show temperature dependency and it is therefore important to map the effects of temperatures observed in hypothermic patients, on drug targets like PDE-enzymes and ABC-transporters [3]. Currently no pharmacological treatment is recommended at temperatures below 30 °C [22]. Thus, to improve the knowledge of hypothermia-induced changes in target molecules that are candidates for providing reduced SVR and positive inotropic effects, we wanted to investigate the effects of isolated hypothermia on cellular elimination of cAMP and cGMP.

2. Materials and methods

2.1. Cell preparation

2.1.1. H9c2 cardiomyocyte cell culture

A clonal H9c2 cardiac muscle cell line, derived from embryonic rat heart tissue, was used [19]. The cells did not contract and were not stimulated electrically during the experiments. The H9c2 cells were kept in a combination of Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin (PenStrep) and 5% CO_2 at 37 °C (former two in combination referred subsequently to as the culture medium). Habitually stored in cryotubes at −196 °C, the cell line was defrosted by warming up to 37 °C in a water bath and afterwards washed with Dimethyl Sulfoxide (DMSO) to secure viability. 10 mL of the culture medium was added, and the cells were centrifugated afterwards for 5 min (800 rpm) in a MSE Minor Centrifuge. Supernatant was removed and additional 15 mL culture medium was added to the cell culture, which was subsequently transferred to 75 cm^3 culture flasks for incubation. After a few days, the cell confluency was sufficient to be transferred to 175 cm^3 culture flasks. This passaging was performed by first removing the culture medium and then thoroughly washing with 10 mL preheated phosphate-buffered saline (PBS). The addition of 1.5 mL preheated 0.25% trypsin-EDTA solution in each flask, followed by 3–5 min of incubation at 37 °C with 5% CO_2, induced detachment of the cells. Successful cell detachment was verified with a light microscope. 8.5 mL culture medium was further added in each flask to end the trypsinization process and supply the cells with nutrients and pH buffering. 1–2.5 mL of the resulting cell suspension was ultimately moved to each of the larger flasks, along with additional culture medium (up to 35 mL). During the cell culture process, a light microscope was used three to four times a week to assess cell density and morphology and the culture medium was replaced regularly to secure stable pH and sufficient nutrient levels. Cell passaging was performed two to three times a week, at a level of 70–80% cell confluency. The range of passage numbers used in the experiments was 9–18.

Before the hypothermia experiment could commence, the cells were aspirated, washed and trypsinated, as described earlier. The cell culture was fully collected from the culture flasks and transferred to a single centrifuge tube (50 mL), in which it was resuspended. Trypan blue (0.4%) was added to 20 μL of the cell culture in a 1:1 ratio and the resulting solution was subsequently assessed by performing a cell count and viability test with a Countess Automated Cell Counter. This was performed in triplicate and thus, the cell concentration was an average of the measurements. The suspension was thereafter diluted with culture medium to obtain a predetermined cell concentration (2.5×10^5–3.5×10^5 cells/well) and distributed to 13 Falcon 6-well Clear Multiwell Plates with 2 mL cell culture in each well. Before the experiment was initiated, the plates were incubated at 37 °C with 5% CO_2 for 48 h.

2.1.2. Inside-out vesicle preparation from red blood cells

Erythrocytes contain both ABCC4 and ABCC5 transporter molecules in their cell membrane and were therefore used for the evaluation of cellular extrusion of cyclic nucleotides during hypothermia [21]. Blodbanken (Department of Immunohematology and Transfusion Medicine, University Hospital of North Norway) provided blood for our second part of the experiment. All participants (n = 35) were randomly assigned, pre-screened and only admitted as donors if they were healthy. The study was performed according to local legislation and institutional requirements included in our agreement with Department of Immunohematology and Transfusion Medicine, University Hospital of North Norway. The regional ethical committee found that ethical review and approval was not required for this study. The participants provided their written informed consent to contribute before sampling at Blodbanken, and we only received anonymized blood samples for the experiments.

Erythrocytes were extracted to prepare so called inside-out vesicles (IOVs) by using a modified version of the Steck IOV preparation, as described earlier [21]. The process was started by collecting and handling fresh EDTA blood at 0 °C–4 °C. The erythrocytes were separated from plasma by centrifugation at 2300*g* for 15 min. Plasma and buffy were disposed of and the remaining cells washed three times with 5 mM Tris- HCl and 113 mM KCl (pH 8.1), and subsequently centrifugated at 1000 g. 5 mM Tris-HCl, 0.5 mM EGTA and 4 mM KCl (pH 8.1) was added to induce lysis and the solution was washed by repeated centrifugation at 20 000 g for 20 min before resuspension in the same buffer. Vesiculation was initiated by adding a hypertonic buffer (0.5 μM Tris-HCl, pH 8.2) to the cell suspension and completed by forcing the solution five times through a syringe needle to promote homogenization of the membranes. The resulting IOVs were separated from right-side vesicles and ghosts by ultracentrifugation (100 000 g) overnight, using a density gradient, ranging from 1.048 g/ml to 1.146 g/ml (Nycodenz, Axis-Shield PoC, Oslo, Norway) in 5 mM Tris, 3 mM KCl, and 0.3 mM, EGTA. This procedure gathered the IOVs in the uppermost band, which was collected, flushed, and resuspended in 1.47 mM KH2PO4, 81 mM K2KPO4 and 140 mM KCl (pH 7.6). Sidedness was verified by using the acetylcholinesterase accessibility test (Ellman GL, 1961). The resulting IOVs, which still contained ABC-transporters in the membrane, enabled us to collect the cyclic nucleotides within for quantification, as it corresponded to the extracellular environment.

2.2. Temperature

In the H9c2-experiments, temperatures 37, 30, 20 °C and exposure to the selected temperatures for 3, 15 or 30 min were studied, using a Labwit Snake 90 incubator (Labwit Scientific). The selected temperatures for the IOV- and PDE-experiments were 37, 34, 32, 28, 24 and 20 °C. The samples were incubated in the designated temperature by using a Grant Optima T100 heated circulating bath (Grant Instruments LTD., Shepreth, England).

2.3. Experimental protocols

2.3.1. Cellular efflux of cyclic nucleotides

IOVs were incubated for 60 min at the designated temperature, with or without 2.0 mM ATP, in the following mixture: 20 mM Tris-HCl, 10 mM MgCl2, 1 mM EGTA, 121 mM KCl. Radioactive labeled [3H]-cAMP or [3H]-cGMP (PerkinElmer, Boston, MA, United States) was added to the incubation solutions in a concentration of 20 μM and 2 μM respectively, depending on the transporter examined. The assay was terminated by adding ice cold buffer (<4 °C), containing 1.47 mM KH2PO4, 8.1 mM K2HPO4 and 140 mM KCl (pH 7.6). The IOVs were subsequently extracted, which was done by filtration through a nitrocellulose

membrane (Bio-Rad Laboratories, Feldkirchen, Germany), and then drying it. The resulting collection of radioactivity upon the filter, was quantified by using a Packard TopCount NXT (Packard, Downers Grove, IL, United states) after adding scintillation fluid (MicroScint-O, PerkinElmer, Groningen, The Netherlands).

2.3.2. Phosphodiesterase enzyme assay

Either 5 μM cAMP or cGMP (Sigma-Aldrich, St. Louis, MO, USA) were used as substrates for PDE3 and PDE5, respectively. The reaction was initiated by adding PDE to the assay solution in designated Eppendorf tubes, containing fresh incubation buffer (10 mM Tris, 8.2 mM Propionic acid, 3 mM Magnesium acetate, 1.5 mM EGTA and 0.5 mg/ml BSA, 0.2 mM DTT), and the selected substrate. The reaction was initiated by adding 0.016 units/μl of PDE3 (Abcam, Cambridge, United Kingdom), or 0.022 units/μl PDE5 (Sigma-Aldrich, St. Louis, United States). Control samples both containing and not containing the PDEs were run in parallel. The incubation time was 30 min at the selected temperature. The reaction was stopped by adding 99.9% methanol to the solutions. Internal standards of cGMP-13C5, cAMP-13C5, AMP-13C5 (Toronto Research Chemicals Inc., Ontario, Canada) and GMP-15N5 (Sigma-Aldrich, St. Louis, MO, USA) in predetermined concentrations were added to each sample, before mass spectrometry (MS) analysis.

2.3.3. Intracellular cAMP quantification in H9c2 cells

Falcon 6-well Clear Multiwell Plates, containing cell culture, were incubated at the selected temperature for either 3, 15, or 30 min. The samples were put on ice afterwards to prevent further intracellular activity. Incubation medium was aspirated, and 2 mL cooled PBS was added to each well for rinsing thoroughly any residuals, followed by aspiration. 25 μL cooled internal standard (cAMP 25 nM) and 50 μL cooled Milli-Q water was added to each sample, as well as 200 μL cooled perchloric acid (8%). Any cells remaining on the well surface were scraped off and investigated with a light microscope. The incubation solutions were transferred to Eppendorf tubes, vortexed and additionally lysed with a probe sonicator (Sonics Vibra-Cell VC130 Ultrasonic Processor, amplitude of 20 μm for 5 s). 200 μL ammonium bicarbonate (2 M) was injected in each tube for neutralization. The solutions were cooled down to −70 °C until next day, before defrosting, vortexing, and centrifugation in a cooled storage room (4 °C) at 13 000 rpm for 10 min 200 μL of the resulting supernatant was transferred from each sample to a 96-position target plate and stored at −70 °C until mass spectrometry measurements were performed.

Cells used for calculating protein concentration, were taken directly from storage in 5% CO_2 and were added 275 μL Milli-Q water (volume corresponding to those of the internal standard, Milli-Q water and perchloric acid) after aspirating the cell medium. Similarly, the cells were scraped, vortexed and sonicated, before an additional 200 μL Milli-Q water was pipetted in each well, as a substitute for the missing volume of ammonium bicarbonate. The plates were stored at −70 °C until protein measurements were performed the day after.

2.4. Mass spectrometry (MS) analysis

Liquid chromatography tandem mass spectrometry (LC-MS) was used to quantify levels of cAMP/AMP and cGMP/GMP from the phosphodiesterase activity experiment, as well as for measuring intracellular levels of cAMP in H9c2 cells. Designated internal standards were added to all samples, as described previously and relevant standard curves were analyzed to determine correct concentrations. There was linearity from 0.2 nM to at least 2000 nM (r2 > 0.998) for cAMP, AMP, and cGMP. For GMP, linearity was present from 2 nM to at least 2000 nM (r2 > 0.998).

2.5. Statistical analysis

To evaluate the cellular efflux of cyclic nucleotides in IOVs, measured radioactive signal was adjusted for protein concentration and sidedness of the IOVs. Intracellular cAMP concentrations in H9c2-cells were also corrected for protein concentration. Statistical analysis was conducted in SigmaPlot 14.0 (Systat Software, San Jose, CA, United States). One-way ANOVA with Holm-Sidak multiple comparisons post hoc test was performed to assess changes in concentrations compared to control temperature (37 °C). ANOVA on ranks with Dunn post hoc test was used when the results were not normally distributed. The results are presented as means ± standard error of mean (SEM), with P-values <0.05 considered as significant results.

3. Results

3.1. cAMP quantification in H9c2 cells

Lowering temperature to severe hypothermia (20 °C) did not decrease cAMP-levels, as compared to normothermia. However, a temperature reduction to moderate hypothermia (30 °C), increased cAMP significantly after 3 and 15 min (3 min at 37 °C: 5.3 ± 0.7 nmol/g vs. 3 min at 30 °C: 7.2 ± 0.2 nmol/g, p = 0.018) and (15 min at 37 °C: 5.9 ± 0.4 nmol/g vs. 15 min at 30 °C: 7.4 ± 0.3 nmol/g, p = 0.027), but not after 30 min of exposure (Fig. 1).

3.2. Cellular efflux

Cyclic nucleotide efflux was registered at all temperatures. There were significantly lower concentrations of cAMP (lower efflux) in IOVs at 28 °C, 24 °C, and 20 °C compared to normothermia (227.58 ± 36.40 nmol/g compared to 97.29 ± 16.45 nmol/g, p = 0.034; 75.87 ± 15.43 nmol/g, p = 0.008; and 25.40 ± 12.35 nmol/g, p < 0.001, respectively) (Table 1, Fig. 2). For cGMP extrusion, significantly lower activity was noticed at 24 °C and 20 °C, compared to 37 °C (77.78 ± 8.98 nmol/g compared to 27.49 ± 5.98 nmol/g, p = 0.012 and 9.71 ± 3.12 nmol/g, p < 0.001, respectively) (Table 1, Fig. 3).

3.3. Phosphodiesterase activity

After 30 min of incubation with PDE3, AMP-levels at normothermia

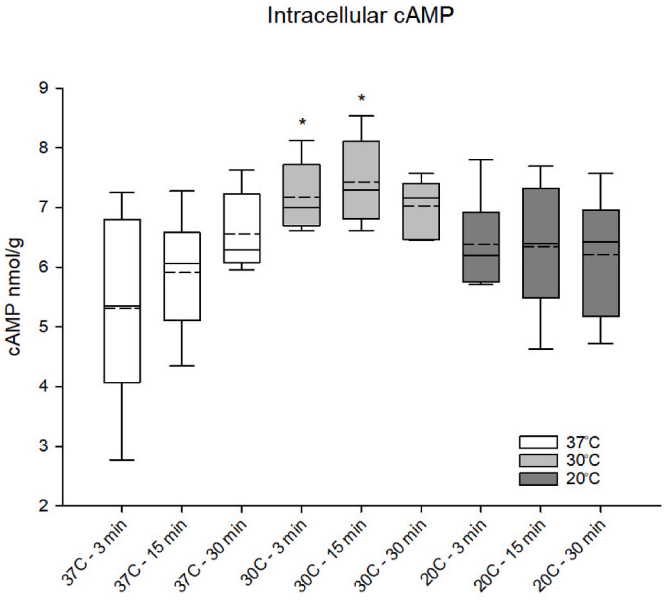

Fig. 1. Intracellular levels of cAMP after 3, 15 or 30 min of incubation in temperatures 37 °C, 30 °C, and 20 °C. The concentrations are presented as means ± SEM and given in nmol/g. * Significant difference (P-value <0.05), when compared to normothermic control.

Table 1
Overview of temperature dependent inhibition of ABCC4, ABCC5, PDE3 and PDE5, in temperatures ranging from 37 °C to 20 °C. * Significant difference (P-value <0.05), when compared to normothermic control.

Temperature	37 °C	34 °C	32 °C	28 °C	24 °C	20 °C
cAMP efflux (nmol/g)	227.58 ± 36.40	247.33 ± 32.99	184.00 ± 22.40	97.29 ± 16.45 *	75.87 ± 15.43 *	25.40 ± 12.35 *
cGMP efflux (nmol/g)	77.78 ± 8.98	61.61 ± 11.30	55.37 ± 14.39	45.06 ± 18.47	27.49 ± 5.98 *	9.71 ± 3.12 *
PDE3 activity (nM AMP)	16.25 ± 2.11	9.19 ± 1,10 *	11.23 ± 2.69	7.20 ± 2.27 *	4.24 ± 1.03 *	4.01 ± 0.74 *
PDE5 activity (nM GMP)	75.01 ± 9.59	57.09 ± 5.06	55.28 ± 3.72	36.43 ± 1.97 *	29.98 ± 2.25 *	21.82 ± 1.92 *

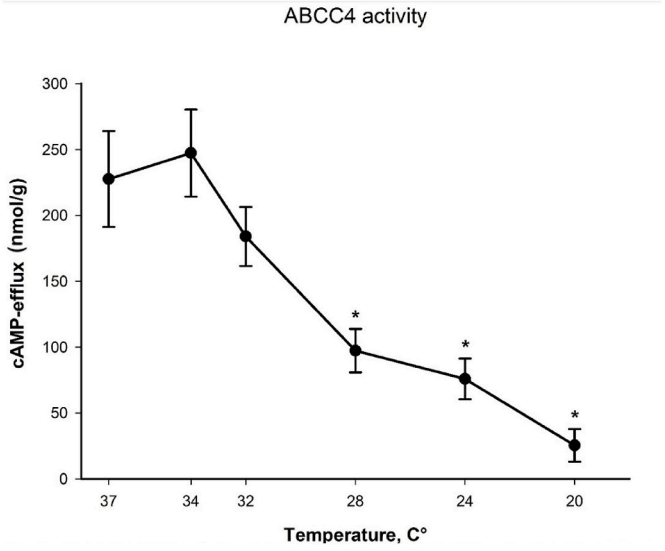

Fig. 2. Temperature dependent effects on cellular efflux of cAMP. The concentrations are presented as means ± SEM and given in nmol/g. * Significant difference (P-value <0.05), when compared to normothermic control.

(37 °C) amounted to 16.25 ± 2.11 nM (Table 1). Temperature reduction had a significant impact on PDE3-activity, showed by reduced AMP-concentrations at all temperatures compared to 37 °C, with exception of 32 °C (34 °C: 9.19 ± 1,10 nM, p = 0.018; 28 °C: 7.20 ± 2.27 nM, p = 0.003; 24 °C: 4.24 ± 1.03 nM, p < 0.001; and 20 °C: 4.01 ± 0.74 nM, p < 0.001) (Fig. 4). At 28 °C, the activity of PDE3 was more than halved. Concerning PDE5 activity, there were significantly lower values of GMP at 28 °C, 24 °C, and 20 °C compared to normothermia (37 °C: 75.01 ± 9.59 nM vs 28 °C: 36.43 ± 1.97 nM, p = 0.023; 24 °C: 29.98 ± 2.25 nM, p = 0.002; and 20 °C: 21.82 ± 1.92 nM, p < 0.001, respectively) (Table 1, Fig. 5).

4. Discussion

This study shows that both moderate and severe hypothermia lead to decreased cellular elimination of cAMP and cGMP by reduced enzyme and transporter activity. Interestingly, higher intracellular levels of cAMP were measured in cultured H9c2 cardiomyocytes at 30 °C (moderate hypothermia) but not at 20 °C (severe hypothermia), indicating that there is a heterogenic effect of moderate hypothermia on production and elimination of cAMP.

Increased cAMP could explain previous in vitro measurements where hypothermia (34–30 °C) had a positive inotropic effect on rodent

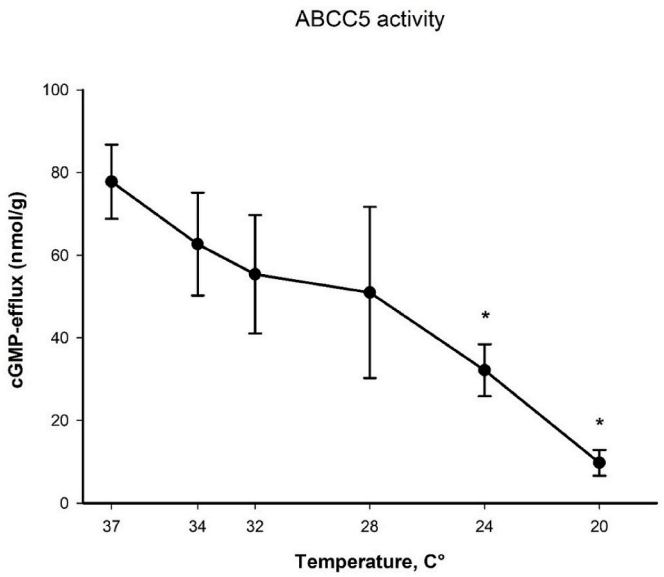

Fig. 3. Temperature dependent effects on cellular efflux of cGMP. The concentrations are presented as means ± SEM and given in nmol/g. * Significant difference (P-value <0.05), when compared to normothermic control.

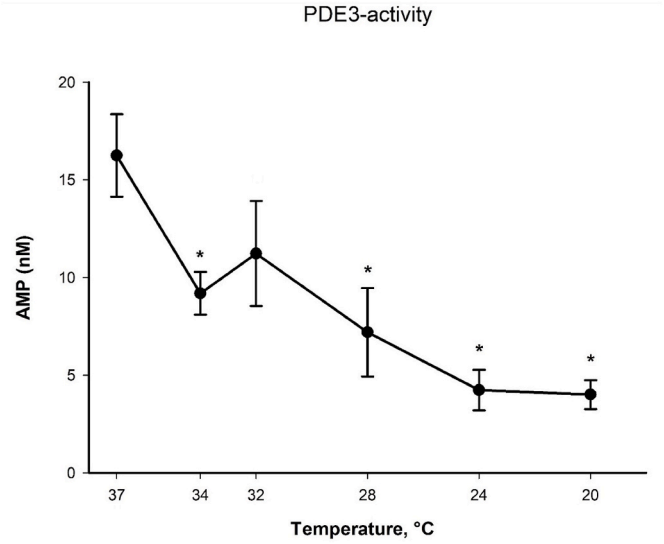

Fig. 4. Temperature dependent effects on PDE3 activity. The concentrations are presented as means ± SEM and given in nM. * Significant difference (P-value <0.05), when compared to normothermic control.

ventricular myocytes [28,30]. Although, when cooling rat ventricular papillary muscles down to 15 °C, before rewarming to 30 °C, a significant reduction in myocardial contractility was seen [15], showing that the pathophysiological changes that cause negative inotropic effect during severe hypothermia are not easily reversed during rewarming. Unfortunately, none of these studies presented concomitant cAMP levels. Some caution must also be taken in evaluation of the cAMP-increase observed in the present study, as the cells were derived from embryonic rat cells and were non-contractile. Other animal studies have reported various findings when stimulating cardiomyocytes with cardioactive drugs affecting cAMP elimination, or more commonly; cAMP production through use of β-receptor agonists or antagonists. As the β1-receptor is considered most important for providing inotropic effect in normothermic conditions, several studies have addressed the pharmacological responses to stimulation of this receptor during

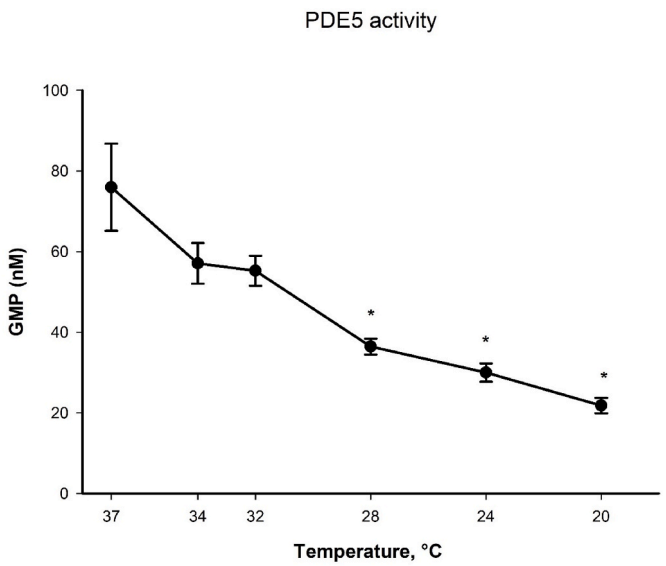

Fig. 5. Temperature dependent effect on PDE5 activity. The concentrations are presented as means ± SEM and given in nM. * Significant difference (P-value <0.05), when compared to normothermic control.

hypothermia. β-receptor agonists increase intracellular cAMP production by receptor-mediated stimulation of adenylyl cyclase (AC) and thereby induce a cascade of intracellular processes that are initiated through cAMP and protein kinase A (PKA). Among these are a positive inotropic effect related to elevated cAMP levels during normothermic conditions. However, it seems that this positive effect on contractile function is attenuated once the core temperature decreases towards severe hypothermia. In one of our studies, β-receptor sensitivity was increased 9-fold in vitro. Further, in vivo epinephrine administration to rats, lead to a four-fold cAMP increase in cardiac tissue at 15 °C but instead of positive hemodynamic effects, the adrenaline-infusion was followed by negative inotropic effects and elevated SVR at this temperature [6]. This correlates with the findings of Mann et al., who found such excessive cAMP levels to be cardiotoxic, through initiating unphysiological increase of cytosolic calcium levels, mediated by increased phosphorylation of L-type calcium channels [24]. The expected cardiac calcium overload was confirmed in several studies by Tveita et al. after prolonged exposure to severe hypothermia in an in vivo rat model and may be aggravated by elevated cAMP production through β-receptor stimulation [20,38]. Through PKA, this could also lead to reduced calcium sensitivity through increased cardiac troponin I (cTnI) phosphorylation [15]. In rats, administering adrenaline concentrations of 0.125 μg/min, gave positive cardiac effects during cooling to 28 °C but such treatment had negative consequences after rewarming, when only rats that had received saline during cooling showed pre-hypothermic hemodynamic responses to adrenaline [34]. This could likely be an effect of elevated cAMP-levels that build up to toxic concentrations and cause down-stream effects, like cTnI-phosphorylation during hypothermia and rewarming, as cellular cAMP-elimination by enzymatic breakdown and efflux is inhibited. Further stimulation of cAMP production after rewarming would therefore be harmful, rather than give a positive inotropic effect before elimination by enzymatic breakdown and transporter-mediated efflux is normalised. Elevated levels of cAMP, independent of pharmacological stimulation, could also be a contributing factor for the increased risk of ventricular arrhythmias, seen at moderate but not severe hypothermia [7,8,10], corresponding to our findings of increased cAMP in cardiomyocytes at 30 °C but not 20 °C in the present study. Hypothermia-induced changes in cyclic nucleotide elimination might however be species-dependent, as cAMP elevation was not evident when examining the effects of the β-receptor agonist isoprotenerol at 22 °C in hamsters [18].

The strategy that is chosen for providing positive hemodynamic effects through cAMP-elevation might also be of importance. Rather than increasing production, inhibition of PDE3-mediated breakdown seems like a favorable approach. In previous in vivo studies, both milrinone and levosimendan (PDE3-inhibitor when administered in high dosages) were shown to mitigate hypothermia-induced cardiac dysfunction by improving stroke volume and reducing SVR in rodents during and after rewarming from 15 °C [4,5]. The positive results were likely a result of both positive inotropic effect and reduced afterload, through the vasodilating properties of PDE3-inhibitors that mediated reduction of SVR. Given these results, later studies targeted SVR alone, to see if afterload-reduction would be sufficient to prevent hypothermia-induced cardiac dysfunction. Hence, sodium nitroprusside, a potent peripheral vasodilator which serves as a source of nitric oxide (NO) and increases cGMP intracellularly in smooth muscle was administered in rats. Both CO and SV increased significantly compared to the control group during rewarming from deep hypothermia [14].

In the present study, we have shown that there is a similar reduction of cellular elimination of both cAMP and cGMP; as we observed a comparable decrease in PDE3 and ABCC4 activity, as in PDE5 and ABCC5 activity. As discussed, reduced cyclic nucleotide elimination might explain the challenges of providing adequate dosing of β-receptor agonists to support cardiovascular function during hypothermia, since the resulting intracellular cAMP-levels could approach toxic levels in response to β-receptor stimulation. PDE3-inhibition by milrinone or levosimendan could be a better strategy in this setting, as it elevates cAMP through inhibition of elimination and might give a more controlled increase in cAMP, not exceeding toxic levels during rewarming [21]. More research is therefore needed to investigate the in vivo effects of PDE3-inhibitors on cytosolic calcium levels and cTnI-phosphorylation during hypothermic conditions. Another approach would be to use low dosages of drugs with a short half-life, that increase cyclic nucleotide production, to allow the clinician swift regulation under tight control of hemodynamic parameters. This is supported by the positive effects of administering sodium nitroprusside in response to changes in mean arterial pressure (MAP) during rewarming from severe hypothermia, with a resulting increase in CO compared to controls [14]. Although the normothermic half-life of sodium nitroprusside and adrenaline are similar (few minutes), hypothermia-induced changes in adrenaline metabolism might complicate use of this drug to alleviate hypothermia-induced cardiac dysfunction. Reduced catechol-O-methyl transferase activity has been suggested to explain a hypothermia-induced hypersensitivity to β-adrenoceptor agonists with resulting intracellular cAMP increase to unphysiological levels [29]. Increased half-life of adrenaline in addition to our findings of reduced PDE3 and ABCC4 activity might therefore have contributed to elevated cAMP levels and catastrophic hemodynamic effects after 5 min of adrenaline administration in severe hypothermia [6].

5. Conclusion

Both moderate and severe hypothermia impairs cellular elimination of cAMP and cGMP through reduced enzymatic breakdown and efflux trough transporter proteins in the cell membrane. Consequently, cardiomyocyte cAMP levels are higher in moderate hypothermia but not in severe hypothermia, indicating a mismatch between production and elimination of cAMP at 30 °C. Our study indicates that treatment of hypothermia-induced cardiac dysfunction should be carefully regulated according to physiological changes in cellular response to the chosen pharmacological strategy.

Data availability statement

Data will be made available upon reasonable request.

Author contributions

AKK, ALS, GS, and ESD planned and designed the research project. AKK, ALS, MF, NS, and TK conducted the laboratory experiments. O-MF analyzed the results using mass spectrometry. The statistical analysis and preparation of the manuscript were carried out by AKK and ESD.

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Paper 2

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ORIGINAL RESEARCH
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Treatment of Cardiovascular Dysfunction with PDE3-Inhibitors in Moderate and Severe Hypothermia—Effects on Cellular Elimination of Cyclic Adenosine Monophosphate and Cyclic Guanosine Monophosphate

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Introduction: Rewarming from accidental hypothermia is often complicated by hypothermia-induced cardiovascular dysfunction, which could lead to shock. Current guidelines do not recommend any pharmacological treatment at core temperatures below 30°C, due to lack of knowledge. However, previous in vivo studies have shown promising results when using phosphodiesterase 3 (PDE3) inhibitors, which possess the combined effects of supporting cardiac function and alleviating the peripheral vascular resistance through changes in cyclic nucleotide levels. This study therefore aims to investigate whether PDE3 inhibitors milrinone, amrinone, and levosimendan are able to modulate cyclic nucleotide regulation in hypothermic settings.

Materials and methods: The effect of PDE3 inhibitors were studied by using recombinant phosphodiesterase enzymes and inverted erythrocyte membranes at six different temperatures—37°C, 34°C, 32°C, 28°C, 24°C, and 20°C- in order to evaluate the degree of enzymatic degradation, as well as measuring cellular efflux of both cAMP and cGMP. The resulting dose-response curves at every temperature were used to calculate IC_{50} and Ki values.

Results: Milrinone IC_{50} and Ki values for cGMP efflux were significantly lower at 24°C (IC_{50}: 8.62 ± 2.69 μM) and 20°C (IC_{50}: 7.35 ± 3.51 μM), compared to 37°C (IC_{50}: 22.84 ± 1.52 μM). There were no significant changes in IC_{50} and Ki values for enzymatic breakdown of cAMP and cGMP.

Conclusion: Milrinone, amrinone and levosimendan, were all able to suppress enzymatic degradation and inhibit extrusion of cGMP and cAMP below 30°C. Our results show that

these drugs have preserved effect on their target molecules during hypothermia, indicating that they could provide an important treatment option for hypothermia-induced cardiac dysfunction.

Keywords: hypothermia, phosphodiesterase 3 inhibitor, phosphodiesterase 3, ATP-binding cassette transporter, cyclic AMP, cyclic GMP, cardiovascular dysfunction

1 INTRODUCTION

Cold-related deaths have been recognized for several hundred years but it was not until late 19th century that hypothermia was defined, when clinical thermometry was made available (Guly, 2011). Today, accidental hypothermia is defined as an unintentional drop in core temperature below 35°C (Brown et al., 2012; Musi et al., 2021). Further, accidental hypothermia is classified in four different stages by The European Resuscitation Council -stage I—*mild hypothermia* (35°C–32°C), stage II—*moderate hypothermia* (32°C–28°C), stage III—*severe hypothermia* (core temperature below 28°C), and stage IV—*severe hypothermia* with no apparent vital signs (core temperature variable) (Lott et al., 2021). Mortality rates approach 40% in severe (<28°C) accidental hypothermia (Vassal et al., 2001; Van der Ploeg et al., 2010). One of the conditions that contribute to the high mortality is hypothermia-induced cardiac dysfunction (HCD) (Han et al., 2010), which is characterized by a decrease in cardiac output, in combination with profound increase in systemic vascular resistance (SVR) when rewarming patients. In worst case scenario, acute cardiovascular failure, so called *rewarming shock,* ensues (Blair, Montgomery and Swan, 1956; Tveita et al., 1996).

Various cardiovascular drugs used to treat heart failure and control blood pressure, target cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). These are intracellular second messengers that are involved in numerous important processes, such as modulating cardiac contraction and vasoregulation. Intracellular levels of these cyclic nucleotides are regulated by the rate of synthesis and degradation by various phosphodiesterases and cellular efflux by transmembrane transporter proteins. Phosphodiesterase 3A (PDE3A), which is especially active in the cardiovascular system, is the main enzyme responsible for degrading cAMP. PDE5A, which resides abundantly in smooth muscle cells in the vascular system, metabolizes primarily cGMP (Omori and Kotera, 2007; Ya et al., 2016). cAMP and cGMP levels are also regulated through active transportation facilitated by transmembrane proteins. These ATP-binding cassette (ABC) transporters are one of the largest transport protein superfamilies (Vasiliou, Vasiliou and Nebert, 2009). ABCC4, which is present in many tissues, transports cAMP out of the cell and has an additional transport site with high Km for both cAMP and cGMP. ABCC5 transports cGMP with high affinity and cAMP with low affinity (Sager and Ravna, 2009).

Well-known β-receptor agonists, such as epinephrine and isoproterenol, increase cAMP levels through the adenylyl cyclase (AC) pathway. Studies in a rat model of severe hypothermia suggest that these drugs increase SVR without simultaneous positive inotropic effect (Dietrichs, Sager and Tveita, 2016) but species-dependent differences exist (Mohyuddin et al., 2021). More promisingly, *in vivo* studies performed in rat models, have demonstrated that PDE3 inhibitors, such as milrinone and levosimendan, have positive effects on stroke volume and cardiac output, almost restoring these parameters to baseline levels during rewarming (Detrichstrichs et al., 2014a; Dettrichstrichs et al., 2014b). Their mechanism of action is through inhibiting cyclic nucleotide breakdown, in contrast to the adrenergic agents, which stimulate their synthesis (Movsesian, 2016). The additional and positive effect of vasodilation by PDE3-inhibitors that was observed in these studies, have prompted research where sodium nitroprusside, a widely known nitric oxide (NO) donor, was studied in the same rat model. The results demonstrate clearly that reducing SVR, without directly supporting the heart, lead to a significant increase in cardiac output (Håheim et al., 2017). These findings indicate that, to treat HCD, it could be favourable to support the failing heart through an alternative route to that of the β-adrenoceptor agonists, and also promote attenuation of SVR. Thus, in the present study we wanted to investigate how PDE3-inhibitors affect cyclic nucleotide regulation in human cells during hypothermia, to improve treatment of severely hypothermic patients.

2 MATERIALS AND METHODS

2.1 Temperature

The chosen temperatures of this study were selected to represent the full spectrum of severity, including normothermia; 37–34—32–28—24–20°C. Temperature was maintained at the chosen level by using a Grant Optima T100 heated circulating bath (Grant Instruments LTD., Shepreth, England). Separate experiments were carried out for all included temperatures and parallels. Exposure to the selected temperature lasted for 30 min.

2.2 Pharmaceuticals

Amrinone (Sigma-Aldrich, Steinheim, Germany), milrinone (United States Pharmacopeia (USP) Reference Standard, Rockville, United States), and levosimendan (Sigma-Aldrich, Steinheim, Germany) were used in seven different concentrations throughout the study, increasing by a factor 10 and ranging from 1.00E-09 to 1.00E-03 M (1 nM–1 mM), respectively.

2.3 Cells

Erythrocytes were chosen for the evaluation of intracellular access of the three drugs as well as cellular extrusion of cyclic nucleotides

during hypothermia, since they contain both ABCC4 and ABCC5 transport molecules in the cell membrane (Köck et al., 2007). Blood was provided by Blodbanken (Department of Immunohematology and Transfusion Medicine, University Hospital of North Norway) where all participants were pre-screened and only admitted as donors if they were healthy. The blood was consistently collected from randomly assigned, pre-screened healthy blood donors. As described in our recent publication from the same project (Selli et al., 2021), the regional ethical committee found that ethical review and approval was not required for this study, as the study was performed according to local legislation and institutional requirements included in our agreement with Department of Immunohematology and Transfusion Medicine, University Hospital of North Norway. The participants provided their written informed consent to contribute before sampling at Blodbanken, and we only received anonymized blood samples for the experiments.

2.4 Experimental Protocols

Three independent experiments were conducted to determine the degree of intracellular access of the substances and cyclic nucleotide turnover. Each medication and control solution were tested in triplicates and at three independent experiments for each temperature. The study protocol has been described previously by our research group (Selli et al., 2021).

2.4.1 Intracellular Access

Fresh (<24 h) EDTA blood from healthy donors (n = 18) was obtained and washed with Krebs-Ringer-Phosphate-Buffer containing glucose (KRPB/G, pH 7.4) and centrifuged (10 min, 1,000 g) three consecutive times. Plasma and buffy coat were removed. KRPB/G was added to the cell solution to bring it to the ratio 1:2.5. Hematocrit (Hct) was measured and adjusted with additional buffer to reach 0.44, in order to give the final Hct of 0.40 in the incubation solution. 500 µl cell suspension (Hct 0.44) were added to test tubes, along with 50 µl of either milrinone (final concentration 10 µM), amrinone (final concentration 100 µM), levosimendan (final concentration 1 µM) or MQ-water (negative control) and incubated for 30 min in the chosen temperature. The reaction was stopped by placing the test tubes on ice and adding 4 ml of ice cold KRFB/G. The samples were subsequently centrifugated (5 min, 600 g), then washed with KRFB/G once more and centrifugation was repeated twice. 50 µl of the remaining red blood cell suspension was transferred to Eppendorf tubes. 50 µl 500 nM IS-Milrinone-d3 was added as an internal standard (TLC Pharmaceutical Standards Ltd.). 200 µl $ZnSO_4$ was added to each test sample, to induce lysis of the cells, and mixed in a vortex mixer. 30 µl of the samples were used for measurement of protein concentration, the rest was mixed with 500 µl acetonitrile and centrifugated (2 min, 13400 g). Finally, 100 µl of the solution was collected for analysis using mass spectrometry (MS).

2.4.2 Cellular Efflux

The transport assay was performed using so called inside-out vesicles (IOVs), which were prepared from erythrocytes by using a modified version of the Steck IOV preparation (Steck, 1974). Freshly collected EDTA blood from healthy donors (n = 35) was prepared at 0°C–4°C, starting by separating the erythrocytes from plasma by centrifugation at 2,300 g for approximately 15 min. Plasma and buffy were disposed of and the remaining cells washed three times with 5 mM Tris- HCl and 113 mM KCl (pH 8.1), and subsequently centrifugated at 1,000 g. Lysis followed thereafter, with ten volumes of 5 mM Tris-HCl, 0.5 mM EGTA, 4 mM KCl (pH 8.1) and washed by repeated centrifugation at 20,000 g for 20 min and resuspension in the same buffer. Vesiculation was initiated by adding 39 volumes of a hypertonic buffer (0.5 µM Tris-HCl, pH 8.2) to one volume of cell suspension and completed by forcing the solution five times through a 27-gauge syringe needle to promote homogenization of the membranes. The resulting IOVs were separated from the right-side vesicles and ghosts by ultracentrifugation (100,000 g) overnight, using a density gradient, ranging from 1.048 g/ml to 1.146 g/ml (Nycodenz, Axis-Shield PoC, Oslo, Norway) in 5 mM Tris, 3 mM KCl, and 0.3 mM, EGTA. This procedure gathered the IOVs in the uppermost band, which was collected, washed and resuspended in 1.47 mM KH_2PO_4, 81 mM K_2KPO_4 and 140 mM KCl (pH 7.6). Sidedness was verified by using the acetylcholinesterase accessibility test (Ellman, 1961). The resulting IOVs, which still contained ABC-transporters in the membrane, enabled us to control the intracellular environment, in this case the surrounding medium, and collect the cyclic nucleotides within. Thus, IOVs were subsequently incubated for 60 min at the designated temperature, with or without 2.0 mM ATP, in the following mixture: 20 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EGTA, 121 mM KCl. Radioactive labeled (^{3}H)-cAMP or (^{3}H)-cGMP (Perkin Elmer, Boston, MA, United States) were added to the incubation solutions in the concentration 20 and 2 µM, respectively, depending on the transporter examined, together with the appointed study drug in concentrations up to 1 mM. The assay was terminated by adding ice cold buffer (<4°C), containing 1.47 mM KH_2PO_4, 8.1 mM K_2HPO_4, and 140 mM KCl (pH 7.6). Next step was to collect the IOVs, which was done by filtration through a nitrocellulose membrane (Bio-Rad Laboratories, Feldkirchen, Germany), and then drying it. The resulting collection of radioactivity upon the filter, was later quantified by using a Packard TopCount NXT (Packard, Downers Grove, IL, United states) after adding scintillation fluid (MicroScint-O, PerkinElmer, Groningen, Netherlands).

2.4.3 Phosphodiesterase Assay

The study drugs were tested upon their ability to hinder cAMP and cGMP hydrolysis by inhibiting PDE3 and PDE5 respectively. Either 5 µM cAMP or cGMP (Sigma-Aldrich, St. Louis, MO, United States) were used as substrates for the enzymes. The reaction was initiated by adding PDE to the assay solution in designated Eppendorf tubes, containing fresh incubation buffer (10 mM Tris, 8.2 mM Propionic acid, 3 mM Magnesium acetate, 1.5 mM EGTA and 0.5 mg/ml BSA, 0.2 mM DTT) selected substrate (cAMP or cGMP) and inhibitor. The reaction was initiated by adding 0.016 units/µl incubate of PDE3 (Abcam, Cambridge, United Kingdom), or 0.022 units/µl incubate of PDE5 (Sigma-Aldrich, St. Louis, United States). Control

samples were free of drug and were either with or without PDE. The incubation time was 30 min in the selected temperature. Reaction was stopped by adding 99.9% methanol to the solutions. Internal standards of cGMP-13C5, cAMP-13C5, AMP-13C5 (Toronto Research Chemicals Inc., Ontario, Canada) and GMP-15N5 (Sigma-Aldrich, St. Louis, MO, United States) were added to each sample, before MS analysis.

2.5 Mass Spectrometry Analysis

Liquid chromatography tandem mass spectrometry (LC-MS) was used to assess concentrations of the inhibitors for experiments performed to determine intracellular access of these drugs, as well as to quantify levels of cAMP/AMP and cGMP/GMP for the PDE3-and PDE5-inhibition plots. Internal standards were used, as described previously. There was a linearity from 0.2 nM to at least 2000 nM ($r^2 > 0.998$) for cAMP, AMP, and cGMP. For GMP, linearity was present from 2 nM to at least 2000 nM ($r^2 > 0.998$). Concerning PDE3-inhibitors, the linearity was found at 10 nM to at least 5,000 nM ($r^2 > 0.99$). Lower limit of quantification (LLOQ) was 0.2 for cAMP, cGMP, and AMP, 2 nM for GMP and 10 nM for PDE3-inhibitors (2 µl injection volume).

2.6 Bioactivity

IC_{50} and K_i values were calculated for each of the drugs, both for their ability to inhibit cAMP- and cGMP- efflux, as well as PDE3 and PDE5 activity. IC_{50} values were derived according to Chou (1976), and K_i values according to Cheng and Prusoff (1973). Inhibition curves were created accordingly, both with and without adjustment for normothermic control.

2.7 Statistical Analysis

Intracellular concentrations of drugs were adjusted for protein concentrations in each sample. The total incubation concentrations were also adjusted for protein concentrations to evaluate the degree of access in percentage. Statistical analysis was performed using one-way ANOVA with Holm-Sidak multiple comparisons post hoc test at each temperature. ANOVA on ranks with Dunn post hoc test was used when the results were not normally distributed. The results are presented as means ± standard error of mean (SEM). p-values < 0.05 were considered significant results. Degrees of freedom (dF) is given as between group values. SigmaPlot 14.0 (Systat Software, San Jose, CA, United States) was used for all analysis, as well as creating the graphs.

3 RESULTS

3.1 Intracellular Access

After 30 min of incubation, all study drugs were found in the IOVs at all temperatures. Decreasing the temperature from 37°C to 20°C did not significantly change their ability to reach their intracellular site of action (**Figure 1**). A higher percentage of levosimendan entered the cells compared to milrinone and amrinone at 34°C (25.06 ± 5.289 vs. 11.41 ± 1.675, ANOVA, dF = 2, F = 12.755, $p = 0.047$, and 2.303 ± 0.284, ANOVA, dF = 2, F = 12.755, $p = 0.007$), as well as at 28°C compared to amrinone (18.29 ± 3.122 vs. 1.66 ± 0.216, ANOVA, dF = 2, F = 23.133, $p = 0.002$). The degree of intracellular access was also significantly higher for milrinone, compared to amrinone, at 28°C (15.06 ± 0.544 vs. 1.66 ± 0.216, ANOVA, dF = 2, F = 23.133, $p = 0.004$).

3.2 Intracellular Elimination by Phosphodiesterase

Milrinone, amrinone, and levosimendan were all able to inhibit both PDE enzymes at all six temperatures. Inhibition plots for PDE3 are depicted in **Figures 2–4**. IC_{50} and K_i values for PDE3 inhibition were not significantly different at hypothermia and the same was true for PDE5 inhibition. IC_{50} and K_i values for PDE3 inhibition by amrinone were significantly higher, regardless of temperature, compared to levosimendan (**Table 2**). At 34°C and 32°C, there were significant differences in values between amrinone and milrinone (IC_{50}: 9.863 ± 1.709 µM vs. 1.771 ± 0.716 µM, ANOVA, dF = 2, F = 23.056, $p = 0.004$ and 15.07 ± 1.855 µM vs. 1.302 ± 0.357 µM, ANOVA, dF = 2, F = 56.783, $p < 0.001$). For PDE5 inhibition, milrinone had consistently higher IC_{50} and K_i values, compared to amrinone (**Table 1**), and they were also significantly higher at 24°C and 20°C, compared to levosimendan (IC_{50}: 253 ± 32.92 µM vs. 148 ± 26.12 µM, ANOVA, dF = 2, F = 10.394, $p = 0.045$ and 330 ± 58.11 µM vs. 157 ± 27.45 µM, ANOVA, dF = 2, F = 9.906, $p = 0.034$).

3.3 Cellular Efflux

The IC_{50} and K_i values for inhibition of cyclic nucleotide extrusion showed no significant differences between 37°C and 28°C for all PDE3 inhibitors. At 24°C and 20°C, however, neither of the study drugs were able to inhibit cAMP-efflux. Milrinone had significantly higher capacity to inhibit cGMP-efflux at 24°C and 20°C, compared to normothermia (IC_{50}: 22.8 ± 1.52 µM compared to 8.62 ± 2.69 µM, ANOVA, dF = 5, F = 5.762, $p = 0.025$ and 7.35 ± 3.51 µM, ANOVA, dF = 5, F = 5.762, $p = 0.014$) (**Table 1**). Amrinone had significantly lower IC_{50} and K_i values at 37°C–34°C for cAMP expulsion and at 34°C–28°C for cGMP-efflux, compared to milrinone (**Table 2**). Levosimendan had significantly higher values at 34°C and 28°C, compared to amrinone, for cGMP-extrusion (**Table 3**).

3.4 Drug Selectivity

3.4.1 Phosphodiesterase Enzymes

Calculated IC_{50} ratios for the phosphodiesterase enzymes showed that notably higher concentrations of all three drugs were needed to inhibit PDE5, compared to PDE3, during normothermia. The same trend was seen at all studied temperatures and appeared to be accentuated for levosimendan from 32°C to 28°C where the IC_{50} ratio for PDE5/PDE3 inhibition doubled from 846 to 1820. Amrinone was the least selective drug with ratios spanning from 10.38 (37°C) to 3.98 (20°C) (**Table 4**).

3.4.2 Cyclic Nucleotide Efflux

Concerning drug selectivity for cGMP/cAMP efflux, milrinone and amrinone appeared to inhibit cGMP extrusion at lower concentrations during normothermic conditions (ratio of

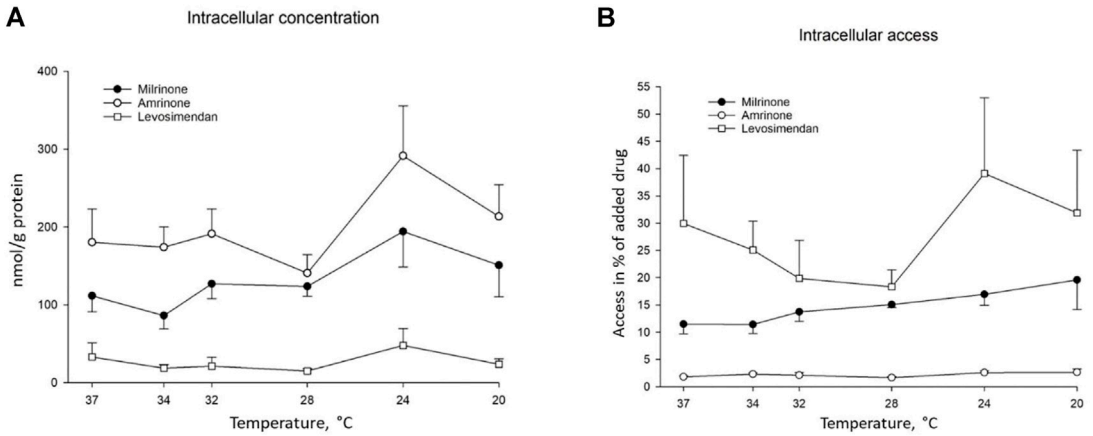

FIGURE 1 | Temperature-dependent intracellular access of added milrinone, amrinone, and levosimendan, presented as means ± SEM **(A)** Intracellular concentration of milrinone, amrinone, and levosimendan in nmol/g protein at temperatures ranging from 37°C to 20°C, *n* = 3. **(B)** Intracellular access of milrinone, amrinone, and levosimendan in % of drug concentration per gram protein, compared to drug concentration per gram protein in the incubation solution at temperatures ranging from 37°C to 20°C, *n* = 3.

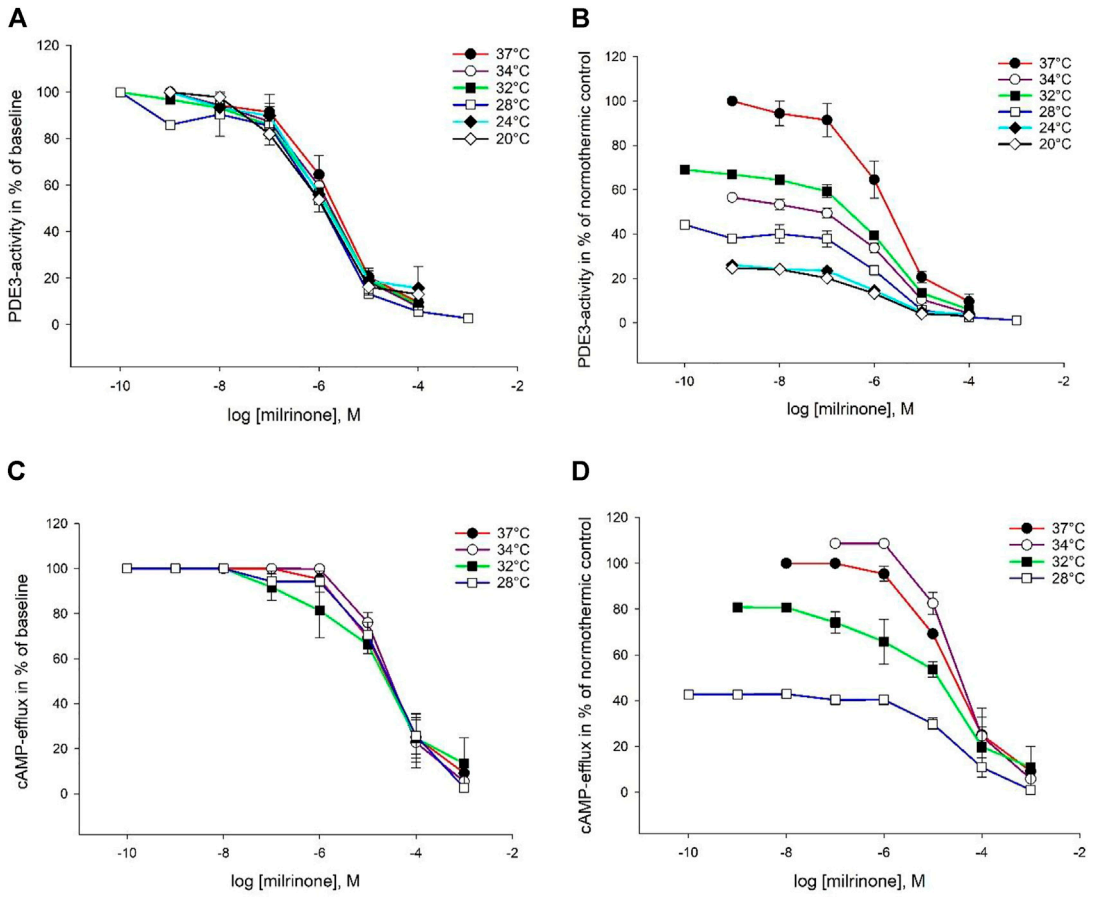

FIGURE 2 | Temperature-dependent inhibition of PDE3 and cAMP-efflux by milrinone. The doses are in logarithm of the concentration in mol/L, presented as means ± SEM. **(A)** Inhibition curves for PDE3-activity at temperatures ranging from 37°C to 20°C. **(B)** Inhibition curves for PDE3-activity in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. **(C)** Inhibition curves for ABCC4-activity at temperatures ranging from 37°C to 20°C. **(D)** Inhibition curves for ABCC4-activity in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C.

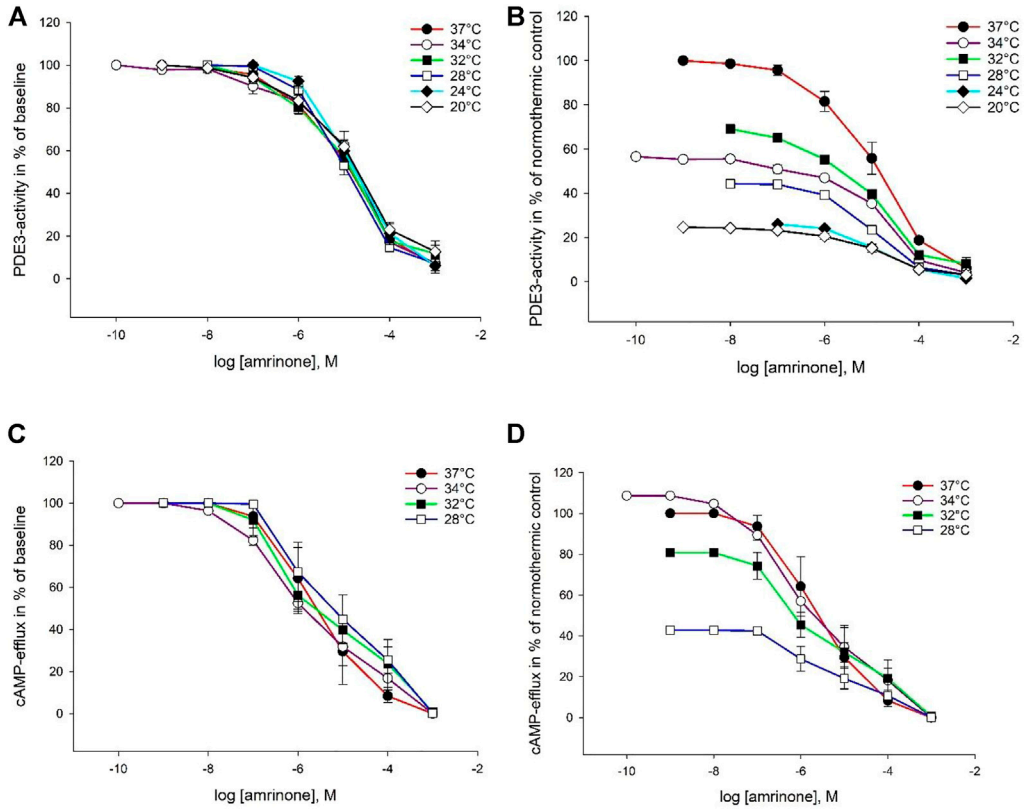

FIGURE 3 | Temperature-dependent inhibition of PDE3 and cAMP-efflux by amrinone. The doses are in logarithm of the concentration in mol/L, presented as means ± SEM. **(A)** Inhibition curves for PDE3-activity at temperatures ranging from 37°C to 20°C. **(B)** Inhibition curves for PDE3-activity in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. **(C)** Inhibition curves for ABCC4-activity at temperatures ranging from 37°C to 20°C. **(D)** Inhibition curves for ABCC4-activity in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C.

0.39 and 0.47, respectively). Inhibition ratio for levosimendan (3.03) indicated an opposite tendency, but when the temperature decreased, the ratio switched to 0.44 at 28°C.

3.4.3 Cyclic Adenosine Monophosphate Elimination

Higher concentrations (IC_{50}) were needed to inhibit ABCC4 compared to PDE3, for milrinone (ratio of 19.66) and levosimendan (28.76) at 37°C. cAMP efflux/PDE3 ratio almost doubled at 34°C for milrinone (37.86) and increased steadily for levosimendan as the temperature fell (270.65 at 28°C). Higher IC_{50} values were consistently registered for inhibiting PDE3-activity by amrinone, compared to ABCC4-function, reflected in an IC_{50}-ratio <1 at all temperatures.

3.4.4 Cyclic Guanosine Monophosphate Elimination

The calculated IC_{50} ratios indicated higher selectivity towards cGMP efflux inhibition than PDE5-mediated cGMP elimination for all medications at all temperatures.

4 DISCUSSION

The present study shows that both levosimendan, milrinone and amrinone are able to reduce cAMP elimination during cooling to severe hypothermia (20°C). All drugs had intact, inhibitory effect on PDE3 and we observed no pharmacodynamic shift towards more potent effect on cGMP-metabolism relative to cAMP metabolism during hypothermic conditions. Our findings therefore suggest that PDE3-inhibition is a promising target to treat and prevent HCD, already from temperatures down to 20°C in severely hypothermic patients.

Current European guidelines for treating accidental hypothermia, do not recommend use of vasoactive drugs in patients with core temperatures below 30°C. As a result, hypothermic cardiac arrest patients should be treated according to the standard advanced life support (ALS) algorithm, but without any pharmacological aid. Once the core temperature reaches >30°C, it is recommended that adrenaline is administered every 6–10 min (instead of 3–5 min, as in normothermic conditions). When the patient is no longer hypothermic, ALS algorithm is followed as originally intended (Lott et al., 2021). In prehospital settings, the focus is to extricate and impede further cooling. Patients with signs of cardiovascular instability, cardiac arrest, or core temperatures <30°C, are transported directly to a hospital with stand-by extracorporeal life support (ECLS) for active rewarming. Remaining patients are triaged to the nearest, appropriate hospital for passive and/or minimally invasive active rewarming (Lott et al., 2021; Paal et al., 2022).

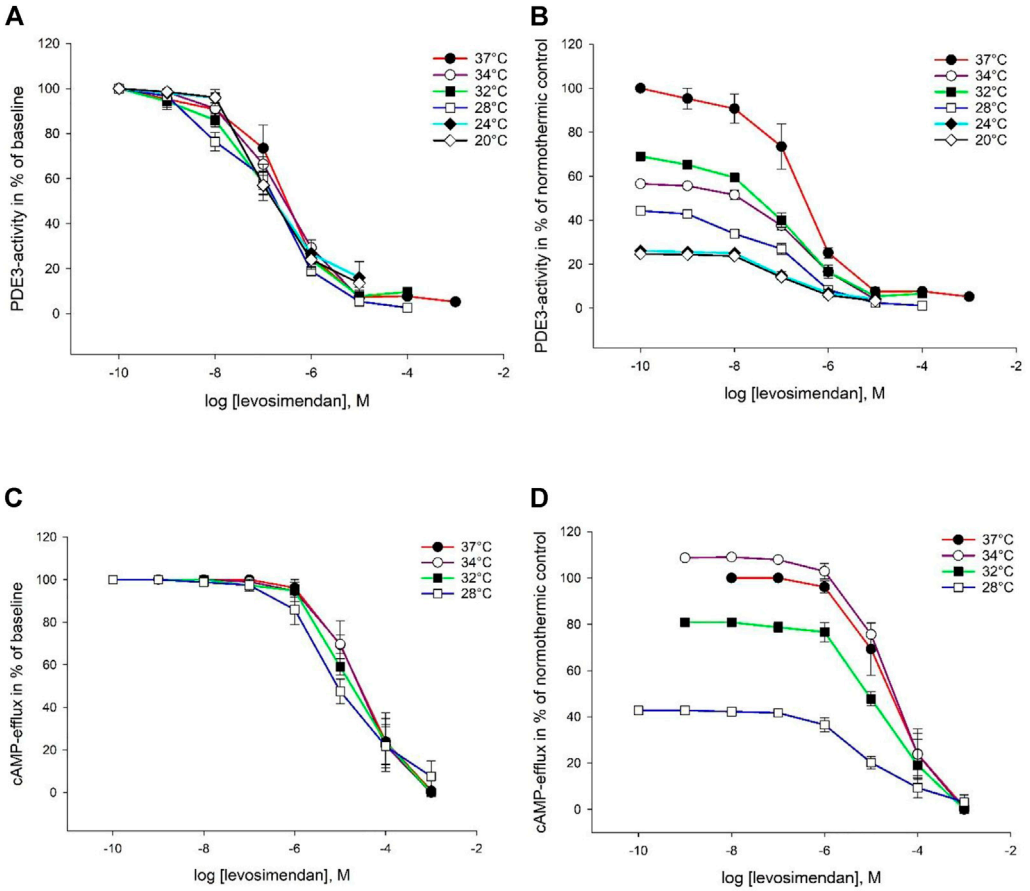

FIGURE 4 | Temperature-dependent inhibition of PDE3 and cAMP-efflux by levosimendan. The does are in logarithm of the concentration in mol/L, presented as means ± SEM. **(A)** Inhibition curves for PDE3-activity at temperatures ranging from 37°C to 20°C. **(B)** Inhibition curves for PDE3-activity in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. **(C)** Inhibition curves for ABCC4-activity at temperatures ranging from 37°C to 20°C. **(D)** Inhibition curves for ABCC4-activity in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C.

TABLE 1 | IC_{50} and K_i values for inhibition of PDE3, PDE5, ABCC4, and ABCC5 at temperatures ranging from 37°C to 20°C.

Milrinone

	PDE3		PDE5		ABCC4		ABCC5	
Temperature (°C)	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**
37	2.99 ± 1.28	0.14 ± 0.059	336 ± 58.60‡	85.3 ± 14.87‡	58.9 ± 13.09‡●	35.69 ± 7.94 ‡●	22.8 ± 1.52	12.91 ± 0.86
34	1.77 ± 0.72 ‡	0.081 ± 0.033‡	255 ± 29.84‡	64.6 ± 7.57‡	67.0 ± 22.65‡	40.65 ± 13.73‡	17,8E ± 1.73‡	10.04 ± 0.96‡
32	1.30 ± 0.36‡	0.060 ± 0.016‡	280 ± 48.33‡	70.9 ± 12.26‡	32.9 ± 14.61	19.95 ± 8.86	17.2 ± 3.02‡	9.70 ± 1.71‡
28	0.82 ± 0.31	0.038 ± 0.014	305 ± 72.47‡	77.4 ± 18.39‡	37.4 ± 24.52	22.67 ± 14.86	18.2 ± 2.03‡●	10.26 ± 1.15‡●
24	1.91 ± 0.73	0.087 ± 0.034	253 ± 32.92‡●	64.2 ± 8.35‡●		-	8.62 ± 2.69#	4.87 ± 1.52#
20	2.15 ± 0.65	0.099 ± 0.030	330 ± 58.11‡●	83.8 ± 14.75‡●		-	7.35 ± 3.51#	4.16 ± 1.98#

#*Significant difference (p-value < 0.05) when compared to normothermic control.*

‡*Significant difference (p < 0.05) when compared to amrinone.*

●*Significant difference (p < 0.05) when compared to levosimendan.*

Values are presented as means ± SEM and given in μM.

In accidental hypothermic patients, one pharmacological strategy to prevent or treat HCD is to elevate cardiac contractility (Dietrichs, Sager and Tveita, 2016). Amrinone, a PDE3-inhibitor, that is, also an important precursor for so-called novel cardiotonic agents, providing positive inotropic effect, has been used intravenously in short-term management of cardiac

TABLE 2 | IC_{50} and K_i values for inhibition of PDE3, PDE5, ABCC4 and ABCC5 at temperatures ranging from 37°C to 20°C.

Amrinone

	PDE3		PDE5		ABCC4		ABCC5	
Temperature	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**
37°C	10.55 ± 3.53●	0.48 ± 0.16●	110 ± 12.80	27.8 ± 3.25	4.65 ± 3.21*	2.82 ± 1,946*	2.20 ± 0.70	1.24 ± 0.39
34°C	9.86 ± 1.71●*	0.45 ± 0.078●*	91.9 ± 3.65	23.3 ± 0.93	1.21 ± 0.10*	0.73 ± 0.058*	2.43 ± 0.75●*	1.37 ± 0.43●*
32°C	15.07 ± 1.86●*	0.69 ± 0.085●*	84.5 ± 5.94	21.5 ± 1.51	5.49 ± 2.25	3.33 ± 1,362	1.75 ± 0.61*	0.99 ± 0.35*
28°C	15.68 ± 2.25●	0.72 ± 0.103●	95.0 ± 7.29	24.1 ± 1.85	8.42 ± 4.81	5.11 ± 2.91	1.10 ± 0.33●*	0.62 ± 0.18●*
24°C	24.87 ± 5.05●	1.14 ± 0.23●	99.1 ± 4.20	25.1 ± 1.07		-	1.10 ± 0.51	0.62 ± 0.29
20°C	26.44 ± 15.73●	1.21 ± 0.72●	105 ± 8.04	26.7 ± 2.04		-	5.44 ± 2.68	3.08 ± 1.52

#Significant difference (p-value < 0.05) when compared to normothermic control.
**Significant difference (p < 0.05) when compared to milrinone.*
●Significant difference (p < 0.05) when compared to levosimendan.
Values are presented as means ± SEM and given in μM.

TABLE 3 | IC_{50} and K_i values for inhibition of PDE3, PDE5, ABCC4, and ABCC5 at temperatures ranging from 37°C to 20°C.

Levosimendan

	PDE3		PDE5		ABCC5		ABCC5	
Temperature	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**	**IC_{50}**	**K_i**
37°C	0.33 ± 0.10‡	0.015 ± 0.00‡	155 ± 4.74	39.2 ± 1.20	9.41 ± 5.91*	5.71 ± 3.58*	28.5 ± 10.29	16.1 ± 5.82
34°C	0.29 ± 0.067‡	0.013 ± 0.00‡	150 ± 13.45	38.0 ± 3.41	12.7 ± 6.60	7.73 ± 4.00	13.9 ± 1.72‡	7.86 ± 0.97‡
32°C	0.19 ± 0.060‡	0 0088 ± 0.00‡	162 ± 22.51	41.2 ± 5.71	16.5 ± 5.56	10.0 ± 3.37	13.2 ± 3.99	7.45 ± 2.25
28°C	0.101 ± 0.026‡	0.0046 ± 0.00‡	183 ± 23.04	46.4 ± 5.85	27.2 ± 21.34	16.5 ± 12.94	11.9 ± 1.35‡*	6.74 ± 0.76‡*
24°C	0.35 ± 0.21‡	0.016 ± 0.01‡	148 ± 26.12*	37.6 ± 6.63*		-	18.4 ± 12.73	10.4 ± 7.20
20°C	0.35 ± 0.200‡	0.016 ± 0.01‡	157 ± 27.45*	39.9 ± 6.97*		-	7.15 ± 1.57	4.04 ± 0.89

#Significant difference (p-value < 0.05) when compared to normothermic control.
**Significant difference (p < 0.05) when compared to milrinone.*
‡Significant difference (p < 0.05) when compared to amrinone.
Values are presented as means ± SEM and given in μM.

TABLE 4 | Drug selectivity for milrinone (M), amrinone (A), and levosimendan (L) at temperatures ranging from 37°C to 20°C. Values are ratios between IC_{50}-values for different elimination ways of cAMP and cGMP. Neither of the drugs inhibited ABCC4-activity below 28°C.

Drug selectivity (IC_{50})/(IC_{50})	(PDE5)/(PDE3) inhibition			(cGMP-efflux)/(cAMP-efflux) inhibition			(cAMP-efflux)/(PDE3) inhibition			(cGMP-efflux)/(PDE5) inhibition		
Temperature	M	A	L	M	A	L	M	A	L	M	A	L
37°C	112	10.38	472	0.39	0.47	3.03	19.66	0.441	28.76	0.068	0.020	0.184
34°C	144	9.32	510	0.27	2.01	1.09	37.86	0.123	43.45	0.070	0.026	0.093
32°C	215	5.61	846	0.52	0.32	0.80	25.27	0.364	85.98	0.061	0.021	0.081
28°C	370	6.06	1820	0.49	0.13	0.44	45.40	0.537	270.7	0.060	0.012	0.065
24°C	133	3.98	427	-	-	-	-	-	-	0.034	0.011	0.124
20°C	154	3.98	450	-	-	-	-	-	-	0.022	0.052	0.045

heart failure (Endoh, 2013). The same pharmacological mechanisms are displayed by milrinone, although it is 30 times more potent than amrinone and it is commonly used in intensive care units as a mean to treat cardiogenic shock (Young and Ward, 1988; Mathew et al., 2021). Levosimendan acts as a PDE3 inhibitor at higher concentrations (>0.3 μM). It is also being used for managing acutely decompensated congestive heart failure (Pathak et al., 2013). Being well-known and widely distributed, these three medications can provide means to treat hemodynamically unstable patients in prehospital settings, especially if the transfer time to nearest ECLS centre is several hours. Nations worldwide without ECLS-facilities are inherently at a disadvantage but can make use of treatment protocols with minimally invasive and cheap pharmacological therapy. Additionally, our findings could also improve more advanced ECLS-treatment of hypothermic patients. Human erythrocytes contain both β_1-and β2-adrenergic receptors, with the latter being predominant (Sager, 1982; Bree et al., 1984). It has been shown that β_2-receptor activation increases the level of cAMP intracellularly, and that there is a concentration-dependent increase in red blood cell filterability when stimulated with adrenaline (Tuvia et al., 1999; Horga et al., 2000). Thus, decreased elimination of cAMP through ABCC4, could provide a pharmacological strategy to increase erythrocyte-

deformability and improve the microcirculation during ECLS-treatment of hypothermic patients.

The evidence for using pharmacological support in cold patients is scarce and the use of, e.g., adrenaline as inotropic support is mainly based on animal studies. The same goes for our study drugs. Various experiments have been conducted, both *in vitro* and *in vivo*. In rodent models, milrinone have a preserved effect on both systolic and diastolic heart function during cooling, down to 15°C and rewarming to 37°C (Tveita and Sieck, 2012; Dietrichs et al., 2014a). On the other hand, in isolated guinea pig hearts, milrinone was shown to have no inotropic effect at study temperatures 31°C and 34°C (Rieg et al., 2009). Interestingly, the latter study demonstrated a temperature-dependent elevation of cardiac inotropy, which could impede any further effect of pharmacological treatment. Studies exploring temperature-dependent effects of levosimendan, show that it has significant positive effects on the circulation in deeply hypothermic rats connected to cardiopulmonary bypass, when compared to adrenaline (Rungatscher et al., 2012). Levosimendan also increases organ blood flow during rewarming, besides contributing to higher CO (Håheim et al., 2020). Knowledge of the effects of amrinone during hypothermia are limited. One study examined whether amrinone could accelerate rewarming in patients after iatrogenic mild hypothermia during neurosurgical procedures (Inoue et al., 2002) but, as of today, no *in vitro* or *in vivo* experiments that investigates potential use of amrinone in accidental hypothermia, exists to our knowledge.

Our findings demonstrate that PDE3-inhibitors milrinone, amrinone, and levosimendan are all able to enter the cells and inhibit PDE3 down to 20°C. Furthermore, we show that their pharmacodynamic effects, in regulating cAMP- and cGMP-elimination, is intact during severe hypothermia and that the dose-response relationship is maintained. It is however likely that the pharmacokinetic properties of these drugs will differ from normothermic temperatures. In general, there is a higher risk of drug and metabolite accumulation in plasma because of diminished clearance at low core temperatures. The volume of distribution is often reduced. This mandates a lower dosage but at the same time, one has to take into account that receptor sensitivity could differ from normothermia (Mallet, 2002; van den Broek et al., 2010). The present study also shows reduced activity of both PDE-enzymes and ABCC-transporters with temperature reduction. Still, all PDE3-inhibitors had intact effect on these target molecules during severe hypothermia. This could provide a more physiological strategy to elevate cAMP levels in hypothermia and avoid an extensive and harmful increase of cAMP, seen with adrenaline-infusion (Dietrichs et al., 2015). Hence, use of PDE3-inhibitors is a promising treatment strategy as their pharmacodynamic effects appear unchanged during hypothermia. Before these drugs are considered for inclusion in guidelines for rewarming accidental hypothermia patients, it is imperative that their temperature-dependent pharmacokinetic properties are established and that their electrophysiological properties are tested in cardiomyocytes. Further, translational studies to assess optimal dosage regimes at different body temperatures and rewarming rates, are therefore necessary to avoid drug toxicity and therapy failure.

5 CONCLUSION

Milrinone, amrinone, and levosimendan are able to cross cell membranes and reach their cytosolic site of action, during severe hypothermia. At temperatures down to 20°C, they have maintained inhibitory effects on inhibiting cellular elimination of cAMP and cGMP, and provide a promising treatment strategy to treat and prevent hypothermia-induced cardiac dysfunction.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AKK, ALS, NS, and TK conducted the experiments in the lab. RAL helped with technical issues and theoretical questions during experiments. O-MF analyzed the results using mass-spectrometry. The results and statistical analysis were interpreted by AKK and ESD. AWR, TT, GS, and ESD planned the research project. AKK, ALS, ESD, O-MF, NS, and GS contributed to the manuscript. All authors read and approved the manuscript.

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Paper 3

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Treatment of Cardiovascular Dysfunction With PDE5-Inhibitors – Temperature Dependent Effects on Transport and Metabolism of cAMP and cGMP

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Introduction: Cardiovascular dysfunction is a potentially lethal complication of hypothermia. Due to a knowledge gap, pharmacological interventions are not recommended at core temperatures below 30°C. Yet, further cooling is induced in surgical procedures and survival of accidental hypothermia is reported after rewarming from below 15°C, advocating a need for evidence-based treatment guidelines. *In vivo* studies have proposed vasodilation and afterload reduction through arteriole smooth muscle cGMP-elevation as a favorable strategy to prevent cardiovascular dysfunction in hypothermia. Further development of treatment guidelines demand information about temperature-dependent changes in pharmacological effects of clinically relevant vasodilators.

Materials and Methods: Human phosphodiesterase-enzymes and inverted erythrocytes were utilized to evaluate how vasodilators sildenafil and vardenafil affected cellular efflux and enzymatic breakdown of cAMP and cGMP, at 37°C, 34°C, 32°C, 28°C, 24°C, and 20°C. The ability of both drugs to reach their cytosolic site of action was assessed at the same temperatures. IC_{50}- and K_i-values were calculated from dose–response curves at all temperatures, to evaluate temperature-dependent effects of both drugs.

Results: Both drugs were able to reach the intracellular space at all hypothermic temperatures, with no reduction compared to normothermia. Sildenafil IC_{50} and K_i-values increased during hypothermia for enzymatic breakdown of both cAMP (IC_{50}: 122 ± 18.9 μM at 37°C vs. 269 ± 14.7 μM at 20°C, $p < 0.05$) and cGMP (IC_{50}: 0.009 ± 0.000 μM at 37°C vs. 0.024 ± 0.004 μM at 32°C, $p < 0.05$), while no significant changes were detected for vardenafil. Neither of the drugs showed significant

hypothermia-induced changes in IC_{50} and K_i values for inhibition of cellular cAMP and cGMP efflux.

Conclusion: Sildenafil and particularly vardenafil were able to inhibit elimination of cGMP down to 20°C. As the cellular effects of these drugs can cause afterload reduction, they show potential in treating cardiovascular dysfunction during hypothermia. As in normothermia, both drugs showed higher selectivity for inhibition of cGMP-elimination than cAMP-elimination at low core temperatures, indicating that risk for cardiotoxic side effects is not increased by hypothermia.

Keywords: hypothermia, PDE5-inhibitors, cyclic AMP, cyclic GMP, afterload reduction, cardiovascular dysfunction, HAPE, ECMO

INTRODUCTION

Accidental hypothermia is associated with a mortality rate up to 40% and is defined as involuntary drop of body core temperature below 35°C (Vassal et al., 2001). Hypothermia-induced cardiac dysfunction (HCD) contributes to the high mortality (Sessler Daniel, 2001; Kondratiev et al., 2006) and is recognized by decreased cardiac output (CO) as well as increased total peripheral resistance (TPR), during hypothermia and rewarming (Mann et al., 1992; Tveita, 2000; Dietrichs et al., 2015). Similarly, cardiovascular failure is associated with a negative outcome during therapeutic hypothermia (Bush et al., 1995). Other critical cardiovascular conditions, like high altitude pulmonary edema (HAPE), could occur in extreme conditions where exposure to low core temperatures are frequent, and evacuation of patients is difficult (Westensee et al., 2013). In such situations, knowledge about the temperature-dependent effect of relevant pharmacological strategies is paramount, to ensure optimal treatment.

Pharmacological manipulation of intracellular cyclic AMP (cAMP) and cyclic GMP (cGMP) levels is used to influence human cardiovascular function during normothermia. Both cAMP and cGMP are intracellular signal molecules with important function in the cardiovascular system. Intracellular levels of cAMP and cGMP are increased by stimulation of the β-receptor-AC-cAMP-PKA and NO-GC-cGMP-PKG pathways, respectively. Elimination is controlled by enzymatic breakdown and cellular extrusion (Subbotina et al., 2017). Elevated cardiomyocyte cAMP-levels are associated with increased cardiac inotropy and chronotropy, while elevation in peripheral smooth muscle cause relaxation and resulting vasodilation. Elevated levels of cGMP in smooth muscle is also associated with peripheral vasodilation, but in cardiomyocytes it has a slightly negative inotropic effect. cAMP degradation in human cardiomyocytes is largely caused by the phosphodiesterase-3A (PDE3A) enzyme. PDE5A is also present and active both in the healthy and failing human heart, as well as in human blood vessels, and is mainly responsible for cGMP degradation (Johnson et al., 2012). Elimination of cAMP and cGMP is also dependent on the activity of cellular efflux pumps. The ATP-binding cassette subfamily-C 4 (ABCC4) is mainly responsible for transporting cAMP out of cells, while cGMP is thought to be removed by ABCC5 (Jedlitschky et al., 2000; Sellers et al., 2012).

Inotropic effects of well-known adrenergic drugs, such as adrenaline and isoprenaline that elevate cAMP through β-receptor stimulation during normothermia, have been explored in rodent models. Hypothermia induced a paradoxical, negative inotropic effect and increased TPR during hypothermia (Dietrichs et al., 2015, 2016), worsening HCD. Several drugs with a different mechanistic approach to affect the β-receptor-AC-cAMP-PKA and NO-GC-cGMP-PKG pathways during hypothermia and rewarming have therefore been investigated *in vivo* (Tveita and Sieck, 2012; Dietrichs et al., 2014a, 2016; Håheim et al., 2017), with diverging hemodynamic effects. Administration of the potent vasodilator sodium nitroprusside lowered TPR, when administered in hypothermic rats. The results showed a positive effect on CO and prevented HCD (Håheim et al., 2017). Similar results were observed after administration of milrinone, a phosphodiesterase-3 (PDE3) inhibitor, impeding enzymatic breakdown of cAMP. In the hypothermic rat, milrinone-infusion resulted in decreased TPR and increased CO (Dietrichs et al., 2014b).

Total peripheral resistance-reduction therefore appears to be a favorable strategy to prevent HCD (Tveita and Sieck, 2012; Dietrichs et al., 2014a, 2018; Håheim et al., 2017). Elevation of cGMP through PDE5-inhibitors like sildenafil and vardenafil, is a potential pharmacological approach in hypothermic patients and a suggested treatment option in HAPE-patients (Luks et al., 2017). Drug specificity during hypothermic conditions is, however, unknown. Therefore, it is important to investigate the ability of sildenafil and vardenafil to reach their site of action and inhibit cGMP, as well as determining whether they also inhibit cAMP elimination at different stages of hypothermia, encountered in critically ill patients. Exploring the temperature-dependent properties of these clinically relevant drugs, could provide important information on their ability to help sustain cardiovascular functions, during hypothermia and rewarming.

MATERIALS AND METHODS

Three different experimental protocols were used to evaluate intracellular access, cellular efflux and phosphodiesterase activity, respectively.

Temperature

According to temperature-dependent clinical signs and physiological changes, The European Resuscitation Council has classified accidental hypothermia into mild hypothermia (35–32°C), moderate hypothermia (32–28°C) and severe hypothermia (below 28°C) (Truhlar et al., 2015). This classification was used in design of the present experiment, where we collected data at relevant temperatures for both accidental and therapeutic hypothermia. Accordingly, we assessed intracellular access of the drugs and pharmacological inhibition of cAMP- and cGMP-efflux, as well PDE3 and PDE5 at 37°C, 34°C, 32°C, 28°C, 24°C, and 20°C.

Pharmacological Substances

Sildenafil (Sigma-Aldrich, Schnelldorf, Germany) and vardenafil (Bayer Pharma AG, Wuppertal, Germany) were used in seven different concentrations increasing by a factor of 10, ranging from 1.00E-09 to 1.00E-03 M (1.00 nM to 1.00 mM), to test their potency at both inhibiting elimination of cAMP and cGMP through reducing cellular efflux and enzyme activity.

Intracellular Access

Both sildenafil and vardenafil are predominantly exerting their effects intracellularly. To estimate whether core temperature reduction would impede their ability to reach the site of action, both drugs were incubated at a concentration of 1.00 μM. The concentration is chosen from therapeutic serum concentrations and were obtained from a systematic literature review in PubMed with (sildenafil) OR (vardenafil) AND (intravenous) AND (plasma concentration) OR (serum concentration) (**Table 1**). References on intravenous administration was chosen due to patients suffering from hypothermia and HAPE often will have reduced consciousness, making oral administration difficult. Further, bioavailability of oral drugs during hypothermia is hard to predict and oral administration is not an alternative for gaining rapid pharmacological effects. The included reference articles had to report adult human data with a relevant cardiovascular topic. Relevant articles from references were also included. For vardenafil, concentrations from intravenous administration were not available and relevant articles for oral administration were included instead. As the aim for this experiment was to detect potential temperature-dependent effects on access through the cell membrane and potential for free fraction of drugs to increase during hypothermia, we chose a concentration of 1.00 μM for both drugs as this corresponded to the highest serum concentrations after intravenous administration of sildenafil.

Blood was provided by Blodbanken UNN (Department of Immunohematology and Transfusion Medicine, University Hospital of North Norway) where all participants (n = 18) were pre-screened and only admitted as donors if they were healthy. Each parallel only included blood from one donor. Experiments were initiated by washing and centrifuging recently (<24 h) drawn EDTA-blood three times with Krebs-Ringer-Phosphate-Buffer containing glucose (KRPB/G, pH∼7.4). The blood was added KRPB/G in a 2.5:1 relationship before measuring hematocrit (Hct) values. Depending on the values, calculations of amount KRPB/G to obtain a Hct of 0.44 were performed, which would later give Hct of 0.40 in the final incubate solution. To start the reaction, 500 μL blood suspension (Hct 0.44) was added to tubes containing 50 μL of either sildenafil, vardenafil or MQ-water (negative control). Each experiment contained triplicates of both drugs and control, and three experiments at each temperature were conducted – in total nine parallels. The reactions were stopped after 30 min (**Table 2**) by putting the tubes on ice and adding 4 mL ice cold KRPB/G. The reaction solutions were washed and centrifugated three times with ice cold KRPB/G. Fifty microliters of the remaining solution was then added to Eppendorf tubes along with 50 μL internal standard (IS), containing 250 nM IS-Sildenafil-d3 and 500 nM IS-Vardenfail-d5 (Toronto Research Chemicals, ON, Canada). Five samples contained 50 μL known concentrations of sildenafil and vardenafil, and 50 μL IS, and served as controls for accurate analysis. All samples were added 200 mL 0.1 M $ZnSO_4$, to lyse the erythrocytes, and then centrifugated. Thirty microliters was taken from Eppendorf tubes for measurements of protein concentration before adding 500 μL acetonitrile. One hundred microliters from each tube was collected for analysis using mass spectrometry (MS).

Cellular Efflux

Cellular extrusion was determined using the inside-out vesicle (IOV) method where erythrocytes from healthy, human donors were sampled. Donors were pre-screened and only admitted as donors by Blodbanken UNN (Department of Immunohematology and Transfusion Medicine, University Hospital of North Norway) if they were healthy. The erythrocytes were separated from plasma by centrifugation and washed. Inside-out vesicles were prepared according to Elin Orvoll et al. (2013) with minor modifications. The membrane vesiculation was initiated by adding hypertonic buffer to the cell suspension. After centrifugation, the suspension was forced through a syringe needle to enhance homogenization of the membranes. IOVs were separated from right side out vesicles (ROV) and unsealed erythrocyte membranes (ghosts) by ultracentrifugation overnight using a density gradient. The uppermost band was collected, washed, and resuspended. Percentage IOV was verified using acetylcholinesterase accessibility test (Ellman et al., 1961). Batches of IOVs used in the parallels were made eight times, including blood from a total of 35 healthy donors.

Inside-out vesicles were then incubated with or without 2 mM ATP and seven different concentrations of sildenafil or vardenafil. The incubation solutions also included radioactive labeled [^{3}H]-cGMP and [^{3}H]-cAMP (Perkin Elmer, Boston, MA, United States), at a concentration of, respectively, 2 μM and 20 μM. The assays were performed in triplicates at three different days: In total nine parallels were performed to calculate results for each concentration of both drugs at all temperatures. Incubation time of 60 min was chosen to ensure sufficient quality of the samples for each parallel (**Table 2**). The transport was stopped by adding ice cold buffer. The IOVs were then filtered through a nitrocellulose membrane (Bio-Rad Laboratories, Feldkirchen, Germany), and the membrane was dried. The dried membranes were

TABLE 1 | Therapeutic plasma concentrations from a literature review of sildenafil and vardenafil, administered for cardiovascular support.

Therapeutic plasma concentration	Calculated concentration in μM	Cardiovascular topic	References	Protein binding	References
Sildenafil	0.449 μM	Pulmonary hypertension	Vachiery et al. (2011)	94–96%	Walker (1999)
	0.101–0.768 μM	Cardiac surgery	Ring et al. (2017)	93–95%	Mehrotra et al. (2007)
Vardenafil	0.010 μM	Pulmonary hypertension	Henrohn et al. (2012)		
	0.044 μM	Pulmonary hypertension	Sandqvist et al. (2013)		

The search was performed in PubMed with (name of drug) AND (intravenous) AND (plasma concentration) OR (serum concentration). As no articles for intravenous vardenafil administration were found, studies conducted after oral administration were accepted instead.

TABLE 2 | Experimental protocol.

	Intracellular access			Enzyme inhibition						Cellular efflux inhibition					
Drug	**Sildenafil**	**Vardenafil**	**Control**	**Sildenafil**		**Vardenafil**		**Control**		**Sildenafil**		**Vardenafil**		**Control**	
Target	Human erythrocyte membranes			PDE3	PDE5	PDE3	PDE5	PDE3	PDE5	cAMP-efflux	cGMP-efflux	cAMP-efflux	cGMP-efflux	cAMP-efflux	cGMP-efflux
Incubation time	30 min			30 min						60 min					
Temperature	37°C–34°C–32°C–28°C–24°C–20°C														

Incubation time of 30 min was chosen for intracellular access and enzyme inhibition, while 60 min was necessary for cellular efflux inhibition to ensure good quality of samples.

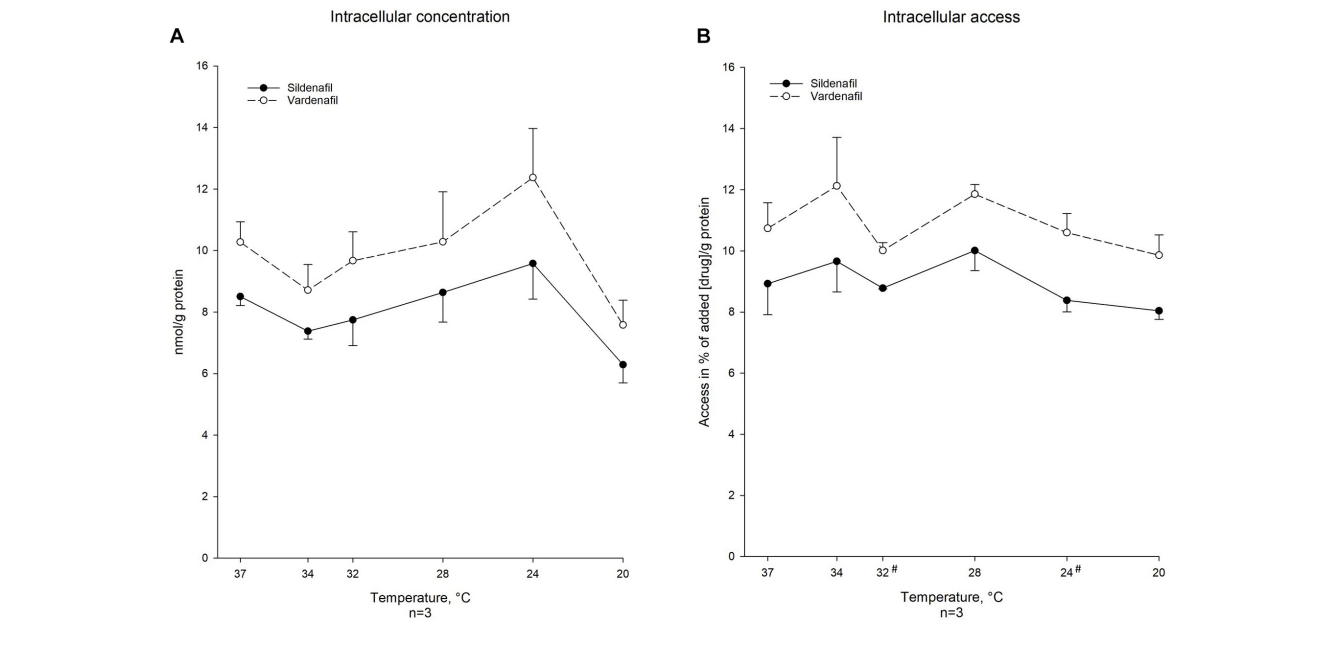

FIGURE 1 | Temperature-dependent intracellular concentration and protein-corrected access in % of added vardenafil and sildenafil. **(A)** Intracellular concentration of sildenafil and vardenafil in nmol/g protein at temperatures ranging from 37°C to 20°C calculated as means ± SEM. Values are calculated from concentration of drug and protein concentration in MS-sample. * Significant difference (*P*-value < 0.05) when nmol/g protein is different from normothermic value. # Significant difference (*P*-value < 0.05) between the two drugs concentrations at specific temperature. **(B)** Intracellular access of sildenafil and vardenafil in % of drug concentration per gram protein in MS-sample compared to drug concentration per gram protein in the incubation solution at temperatures ranging from 37°C to 20°C. Values are in means ś SEM. * Significant difference (*P*-value < 0.05) when % is different from normothermia. # Significant difference (*P*-value < 0.05) between the two drugs access (in %) at specific temperature.

later added scintillation fluid and radioactivity was measured using a Packard TopCount NXT (Packard, Downers Grove, IL, United States).

Experiments determined total ATP-dependent cellular efflux of cGMP or cAMP from IOVs. As described in previous studies, ABCC5 and ABCC4 are the dominant efflux pumps for cGMP and cAMP (Jedlitschky et al., 2000; Sellers et al., 2012), respectively.

Phosphodiesterase Activity

Ability of drugs to inhibit cAMP and cGMP hydrolysis by PDE3 and PDE5, respectively, was tested by incubating 5 μM cAMP

TABLE 3 | Linear regression analysis for intracellular access and inhibition of phosphodiesterase-5 (PDE5), phosphodiesterase-3 (PDE3), and inhibition of cAMP- and cGMP-efflux calculated at 37°C, 34°C, 32°C, 28°C, 24°C, and 20°C.

Regression analysis	**Intracellular access**		**PDE5-inhibition**		**PDE3-inhibition**		**Inhibition of cGMP-efflux**		**Inhibition of cAMP-efflux**	
Drug	**Sildenafil**	**Vardenafil**	**Sildenafil**	**Vardenafil**	**Sildenafil**	**Vardenafil**	**Sildenafil**	**Vardenafil**	**Sildenafil**	**Vardenafil**
Equation	$y = 7.14 + 0.063x$	$y = 9.14 + 0.059x$	$y = 0.064 - 0.002x$	$y = 0.039 - 0.001x$	$y = 367 - 7.90x$	$y = 131 - 3.09x$	$y = -2.07 + 19.2x$	$y = 9.64 - 0.098x$	$y = -7.13 + 0.363x$	$y = 26.4 - 0.545x$
Pearson's *r*	0.535	0.384	−0.906	−0.921	−0.751	−0.946	0.699	0.224	0.931	0.598
P-value	0.21	0.35	<0.01	<0.01	<0.01	<0.01	0.05	0.51	0.13	0.28

The y-value for the intracellular access equation is added [drug]/g protein and the y-value for inhibition equations are concentration of drug in μM. The x-value is temperature in degrees Celsius for all equation. Pearson's r is added to depict how well the regression fitted the observations.

or cGMP (Sigma-Aldrich, St. Louis, MO, United States) with the seven different concentrations of sildenafil and vardenafil. The assays were performed in triplicates at three different days. A total of nine parallels were used to calculate results for each concentration of both drugs at all temperatures. The reaction was started by adding either a solution containing 0.016 units/μg protein of PDE3 (Abcam, Cambridge United Kingdom), or 0.022 units/μg protein of PDE5 (Sigma-Aldrich, St. Louis, MO, United States), to the Eppendorf tubes. Parallels for cAMP-metabolism included PDE3 only and parallels for cGMP-metabolism included PDE5 only. Control samples were free of drug and was either with or without PDE3 or PDE5. This was done to assure that only the relevant PDE was responsible for breakdown of the cyclic nucleotide, as no other enzyme nor cellular material was added to the incubations. The incubation time was 30 min (see **Table 2**). Reaction was stopped by adding methanol to the tubes. Internal standard of cGMP/GMP or cAMP/AMP (Sigma-Aldrich, St. Louis, MO, United States, Germany and Toronto Research Chemicals Inc., Toronto, ON, Canada) were added to each sample. Five samples contained only known concentrations of cGMP/GMP or cAMP/AMP and served as calibrators. Samples were analyzed for cGMP/GMP and cAMP/AMP content, using MS.

Mass Spectrometry (MS) Analysis

Quantification of cAMP/AMP, cGMP/GMP, and PDE5-inhibitors in PDE- and intracellular access experiments were performed with liquid chromatography tandem mass spectrometry (LC-MS/MS). Preparation of samples for LC-MS/MS-analysis is described in the relevant paragraphs above. The method was found to be linear from 0.2 nM to at least 2000 nM ($r^2 > 0.998$) for cAMP, cGMP, and AMP. For GMP the method was linear from 2 nM to at least 2000 nM ($r^2 > 0.998$), and 10 nM to at least 5000 nM for the PDE5-inhibitors ($r^2 > 0.998$). Lower limit of quantification (LLOQ) was found to be 0.2 nM for cAMP, cGMP, and AMP, 2 nM for GMP and 10 nM for the PDE5-inhibitors (2 μl injection volume).

Data Analysis

The ability of drugs to inhibit cAMP-efflux, cGMP-efflux, PDE3, and PDE5 were determined by calculating IC_{50}- and K_i-values from inhibition plots. IC50 values were calculated according to Chou (1976) and data were transformed to K_i-values according to Cheng and Prusoff (1973). IC_{25} and IC_{75} values were estimated by polynomial, cubic regression, based on the inhibition curve of each experiment. Measurement of intracellular concentrations of drugs were adjusted for protein concentrations in each sample. The incubation concentrations were also adjusted for protein concentration in each sample to evaluate the access in percentage. Results are presented as mean ± standard error of mean (SEM). A one-way ANOVA with Holm–Sidak multiple comparisons *post hoc* test was used to compare the IC_{50} and K_i-values for the drugs, as well as intracellular concentrations of drugs, at each temperature with normothermic baseline (37°C). When results were not normally distributed, ANOVA on ranks was used with a Dunn *post hoc* test. Two-tailed *t*-tests were performed to compare IC_{50}-values, and adjusted

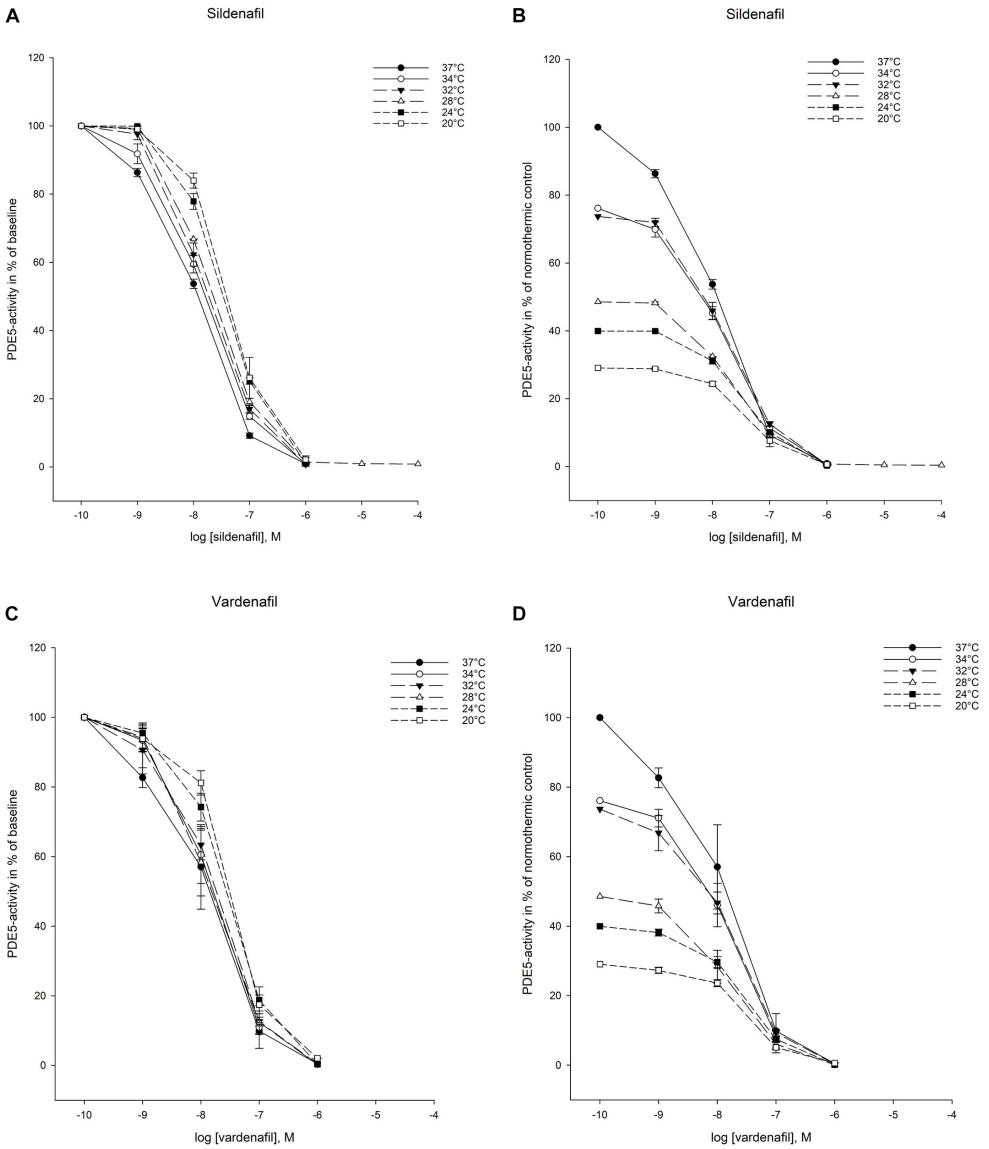

FIGURE 2 | Temperature-dependent inhibition of phosphodiesterase-5 (PDE5) by vardenafil and sildenafi. **(A)** Sildenafil inhibition curves for PDE5-activity at temperatures ranging from 37°C to 20°C. The doses of sildenafil are in logarithm of the concentration in mol/L. **(B)** Inhibition curves for PDE5-activity by sildenafil in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. The doses of sildenafil are in logarithm of the concentration in mol/L. **(C)** Vardenafil inhibition curves for PDE5-activity at temperatures ranging from 37°C to 20°C. The doses of vardenafil are in logarithm of the concentration in mol/L. **(D)** Inhibition curves for PDE5-activity by vardenafil in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. The doses of vardenafil are in logarithm of the concentration in mol/L.

intracellular concentrations, of sildenafil and vardenafil at each temperature. Regression analysis was performed to evaluate whether a linear relationship existed between IC_{50}-values and temperature for each drug at each elimination pathway of cAMP and cGMP. Regression analysis was also performed to evaluate if there was a linear relationship between intracellular access of the two drugs and temperature. Pearson's r was calculated for every regression analysis to evaluate how well the calculated lines fitted the observations. P-values < 0.05 were considered significant for our data analysis. All analysis were performed using SigmaPlot 14.0 (Systat Software, San Jose, CA, United States, RRID:SCR_003210).

RESULTS

Intracellular Access of Drugs During Hypothermia

Sildenafil and vardenafil, at incubate concentration of 1.00 μM, were able to reach the cytosol at all included temperatures from

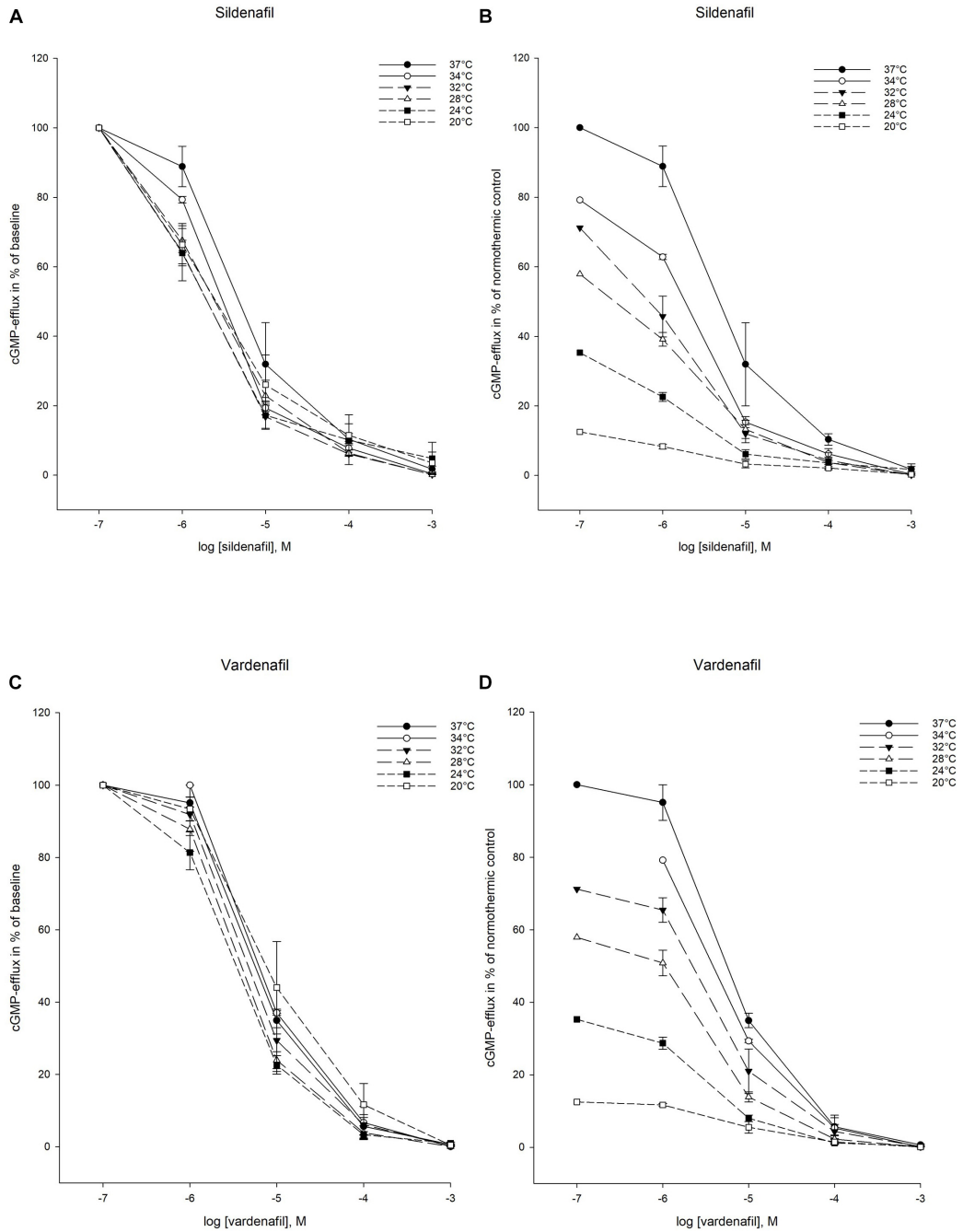

FIGURE 3 | Temperature-dependent inhibition of cGMP-efflux in inside-out vesicles (IOV). **(A)** Sildenafil inhibition curves for ABCC5-activity at temperatures ranging from 37°C to 20°C. The doses of sildenafil are in logarithm of the concentration in mol/L. **(B)** Inhibition curves for ABCC5-activity by sildenafil in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. The doses of sildenafil are in logarithm of the concentration in mol/L. **(C)** Vardenafil inhibition curves for ABCC5-activity at temperatures ranging from 37°C to 20°C. The doses of vardenafil are in logarithm of the concentration in mol/L. **(D)** Inhibition curves for ABCC5-activity by vardenafil in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. The doses of vardenafil are in logarithm of the concentration in mol/L.

37°C to 20°C after 30 min incubation (**Figure 1**). Decreased temperature did not affect the ability of either drug to reach their intracellular site of action. A significantly smaller percentage of sildenafil was able to reach the intracellular space compared to vardenafil at 32°C (% of drug: 8.78 ± 0.027 vs. 10.0 ± 0.257, $p < 0.05$) and at 24°C (% of drug: 8.38 ± 0.381 vs. 10.6 ± 0.625, $p < 0.05$). Regression analysis showed no significant linear relationship between access, neither when looking at total

amount nor percentage that reached cytosol, and temperature for any of the drugs (**Table 3**).

Cellular Elimination of cGMP

Both sildenafil and vardenafil were able to inhibit cellular elimination of cGMP by PDE5 and efflux pumps at all temperatures (**Figures 2, 3**).

Intracellular Elimination by PDE5

IC_{50}- and K_i -values for PDE5 inhibition were significantly increased compared to normothermic baseline at 32°C for sildenafil (IC_{50}: 0.024 ± 0.004 vs. 0.009 ± 0.000 μM, $p < 0.05$) and remained elevated down to 20°C, with exception of measurements at 28°C (**Tables 4, 5**). For vardenafil, IC_{50}-values did not significantly differ from normothermic control at any temperatures. When comparing IC_{50}- and K_i-values of sildenafil and vardenafil at different temperatures, sildenafil had significantly higher IC_{50}- and K_i -values at 20°C (IC_{50}: 0.037 ± 0.003 vs. 0.023 ± 0.002 μM, $p < 0.05$). The IC_{25}–IC_{75} interval was significantly increased for sildenafil compared to normothermic baseline at 24°C (IC_{25}–IC_{75}: 0.098 ± 0.012 μM vs. 0.038 ± 0.000 μM, $p < 0.05$) and 20°C (IC_{25}–IC_{75}: 0.132 ± 0.048 μM 20°C vs. 0.038 ± 0.000 μM, $p < 0.05$) (**Table 6**).

The temperature-dependent increase in IC_{50} for both sildenafil and vardenafil appeared to follow a linear pattern (**Table 3**). Regression analysis provided the equation for calculating sildenafil IC_{50} values during hypothermia: $y = 0.064 - 0.002x$ μM, with x being the temperature in Celsius. The R-value was −0.906 ($p < 0.05$). For vardenafil the calculated equation was $y = 0.039 - 0.001x$ μM, with $R = -0.921$ ($p < 0.01$).

Cellular Efflux

For ABBC5 inhibition, there were no statistically significant difference in IC_{50}- and K_i-values for either sildenafil or vardenafil when compared to 37°C (**Tables 4, 5**). Only at 32°C there was a significant lower IC_{50}- and K_i-value for sildenafil than vardenafil (IC_{50}: 3.68 ± 0.416 vs. 9.40 ± 0.762 μM, $p < 0.05$). The IC_{25}–IC_{75} interval remained stable for both drugs during hypothermia.

Regression analysis showed that neither sildenafil nor vardenafil inhibition of cGMP-efflux followed a linear pattern during temperature reduction (**Table 3**).

Cellular Elimination of cAMP

PDE3-mediated elimination of cAMP was inhibited by both sildenafil and vardenafil at all temperatures in the experimental protocol (**Figure 4**). Inhibition of cAMP-efflux was however only achieved down to 28°C (**Figure 5**). At the two lowest temperatures, 24°C and 20°C, neither of the drugs were able to inhibit cAMP-efflux. These temperatures are therefore excluded from IC_{50} and K_i calculations.

Intracellular Elimination by PDE3

IC_{50}- and K_i-values for PDE3-inhibition was substantially increased for sildenafil at 20°C compared to normothermic control (IC_{50}: 269 ± 14.7 vs. 122 ± 18.9 μM, $p < 0.05$). For vardenafil, there were no significant differences compared to normothermia. Differences in IC_{50}- and K_i-values between drugs, $p < 0.05$, were observed at all temperatures except 24°C (**Tables 4, 5**). The IC_{25}–IC_{75} interval was significantly increased for both sildenafil and vardenafil compared to normothermic baseline at 20°C (IC_{25}–IC_{75} sildenafil: 697 ± 22.5 μM vs. 460 ± 38.8 μM, $p < 0.05$. IC_{25}–IC_{75} vardenafil: 790 ± 105 μM vs. 61.0 ± 14.3 μM, $p < 0.05$) (**Table 6**).

Regression analysis showed significant correlation ($p < 0.05$) between IC_{50} values and temperature for sildenafil with $y = 367 - 7.90x$ μM, $R = -0.751$. For vardenafil, there was a significant correlation ($p < 0.05$) with $R = -0.946$, providing the equation $y = 131 - 3.09x$ μ M (**Table 3**).

Cellular Efflux

The IC_{50}- and K_i-values for inhibition of cAMP-efflux by sildenafil and vardenafil at hypothermic temperatures, showed no significant difference when compared to normothermic control. When comparing drugs however, IC_{50}- and K_i-values for sildenafil were significantly lower than vardenafil at 32°C (IC_{50}: 4.66 ± 1.37 vs. 13.0 ± 1.69 μM, $p < 0.05$) (**Tables 4, 5**). The IC_{25}–IC_{75} interval remained stable for both drugs during hypothermia.

Regression analysis showed no significant correlation for IC_{50} pattern with temperature for neither of the drugs (**Table 3**).

Drug Selectivity

Phosphodiesterase Enzymes

As expected, calculated ratios between IC_{50} values showed that the sildenafil concentration needed to inhibit PDE3 was higher (by a factor of 14400) than the dose needed to achieve PDE5-inhibition during normothermic conditions (37°C). Vardenafil was less selective, with a PDE3/PDE5-inhibiting ratio of 1960. Hypothermia appeared to reduce PDE5-selectivity of sildenafil, with a PDE3/PDE5-inhibiting ratio of 7270 at 20°C and increase PDE5-selectivity of vardenafil, as the PDE3/PDE5-inhibiting ratio was increased to 3230 at 20°C (**Table 7**).

Efflux Pumps

The normothermic cAMP-efflux/cGMP-efflux IC_{50}-ratio for sildenafil (0.832) and vardenafil (1.11) indicate that both drugs inhibit cAMP and cGMP efflux at similar concentrations. The tendency for both drugs is a modest increase in selectivity for cGMP-efflux during hypothermia, with a sildenafil-ratio of 0.917 and vardenafil-ratio of 1.95 at 20°C (**Table 7**).

cGMP Elimination

During normothermic conditions, the predominant inhibition of cGMP-elimination by sildenafil and vardenafil, is through PDE5-inhibition, as the cGMP-efflux/PDE5 inhibition-ratio was 815 and 625, respectively. For both drugs the ratios decreased during hypothermia

TABLE 4 | IC_{50}-values for inhibition of phosphodiesterase-5 (PDE5), phosphodiesterase-3 (PDE3), and inhibition of cAMP- and cGMP-efflux at temperatures ranging from 37°C to 20°C.

IC_{50}	PDE5		PDE3		cGMP-efflux		cAMP-efflux	
Temperature	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)
37°C	0.009 ± 0.000	0.008 ± 0.003	122 ± 18.9#	16.5 ± 6.08#	6.96 ± 2.58	5.26 ± 1.46	5.79 ± 1.73	5.85 ± 0.51
34°C	0.014 ± 0.002	0.015 ± 0.005	91.9 ± 12.6#	24.9 ± 5.73#	3.68 ± 0.416#	9.40 ± 0.762#	5.91 ± 2.22	5.82 ± 0.685
32°C	0.024 ± 0.004*	0.015 ± 0.004	103 ± 14.1#	40.5 ± 3.78#	2.53 ± 0.878	6.50 ± 1.20	4.66 ± 1.37#	13.0 ± 1.69#
28°C	0.017 ± 0.003	0.015 ± 0.003	95.7 ± 16.1#	32.7 ± 11.0#	2.98 ± 0.304	4.94 ± 0.891	2.73 ± 0.382	9.63 ± 5.79
24°C	0.028 ± 0.005*	0.022 ± 0.003	143 ± 44.8	55.5 ± 4.75	2.21 ± 0.434	3.75 ± 0.926	–	–
20°C	0.037 ± 0.003*#	0.023 ± 0.002#	269 ± 14.7*#	73.4 ± 26.3#	2.76 ± 1.27	10.9 ± 4.79	–	–

Values are mean ± SEM and given in ±M.
**Significant difference (P-value < 0.05), when compared to normothermic control.*
#Significant difference (P-value < 0.05) when IC_{50} values of sildenafil and vardenafil are compared at same temperature. Neither of the drugs inhibited ABCC4-activity below 28°C.

TABLE 5 | K_i-values for inhibition of phosphodiesterase-5 (PDE5), phosphodiesterase-3 (PDE3), and inhibition of cAMP- and cGMP-efflux at temperatures ranging from 37°C to 20°C.

K_i	PDE5-inhibition		PDE3-inhibition		cGMP-efflux		cAMP-efflux	
Temperature	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)
37°C	0.002 ± 0.000	0.002 ± 0.001	5.61 ± 0.865#	0.757 ± 0.276#	3.93 ± 1.46	2.97 ± 0.823	3.51 ± 1.05	3.55 ± 0.311
34°C	0.004 ± 0.000	0.004 ± 0.001	4.21 ± 0.579#	1.14 ± 0.263#	2.08 ± 0.235#	5.31 ± 0.431#	3.59 ± 1.35	3.53 ± 0.415
32°C	0.006 ± 0.001*	0.004 ± 0.001	4.66 ± 0.647#	1.85 ± 0.173#	1.43 ± 0.497	3.67 ± 1.13	2.83 ± 0.828#	7.86 ± 1.02#
28°C	0.004 ± 0.001	0.004 ± 0.001	4.38 ± 0.739#	1.50 ± 0.505#	1.68 ± 0.172	2.79 ± 0.504	1.66 ± 0.232	5.84 ± 3.51
24°C	0.007 ± 0.001*	0.006 ± 0.001	6.49 ± 2.05	2.54 ± 0.218	1.25 ± 0.245	2.12 ± 0.523	–	–
20°C	0.009 ± 0.001*#	0.006 ± 0.000#	12.3 ± 0.675*#	3.36 ± 1.21#	1.56 ± 0.718	6.17 ± 2.71	–	–

Values are mean ± SEM and given in μ M.
**Significant difference (P-value < 0.05) when compared to normothermic control.*
#Significant difference (P-value < 0.05) when IC50 values of sildenafil and vardenafil are compared at same temperature. Neither of the drugs inhibited cAMP-efflux below 28°C.

TABLE 6 | IC_{25}–IC_{75} intervals, describing the concentration needed to increase inhibition of phosphodiesterase-5 (PDE5), phosphodiesterase-3 (PDE3), and inhibition of cAMP- and cGMP-efflux from 25% to 75%, at temperatures ranging from 37°C to 20°C.

IC_{25}–IC_{75}	PDE5		PDE3		cGMP-efflux		cAMP-efflux	
Temperature	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)	Sildenafil (μM)	Vardenafil (μM)
37°C	0.038 ± 0.000	0.059 ± 0.029	460 ± 38.8	61.0 ± 14.3	23.6 ± 9.08	12.6 ± 3.89	27.1 ± 9.36	30.9 ± 6.82
34°C	0.055 ± 0.004	0.028 ± 0.012	411 ± 40.4	72.1 ± 3.24	13.5 ± 1.74	19.2 ± 3.16	19.8 ± 1.82	19.9 ± 0.967
32°C	0.060 ± 0.006	0.062 ± 0.016	485 ± 32.4	107 ± 27.0	9.76 ± 3.99	18.0 ± 4.12	20.0 ± 4.27	27.3 ± 6.19
28°C	0.083 ± 0.008	0.049 ± 0.016	450 ± 35.9	128 ± 35.9	12.0 ± 1.96	10.6 ± 2.75	13.8 ± 3.85	17.3 ± 6.59
24°C	0.098 ± 0.012*	0.074 ± 0.005	584 ± 55.0	148 ± 31.9	11.4 ± 4.73	13.0 ± 1.74	–	–
20°C	0.132 ± 0.048*	0.103 ± 0.027	697 ± 22.5*	709 ± 105*	19.2 ± 8.45	28.1 ± 14.6	–	–

Values are mean ± SEM given in μ M.
**Significant difference (P-value < 0.05) when compared to normothermic control. Neither of the drugs inhibited cAMP-efflux below 28°C.*

to 170 for vardenafil at 24°C and 74.5 for sildenafil at 20°C (**Table 7**).

cAMP Elimination

Both sildenafil and vardenafil are more efficient inhibitors of cAMP efflux than PDE3-mediated elimination in normothermic conditions, with an cAMP-efflux/PDE3 inhibition-ratio of 0.047 for sildenafil and 0.354 for vardenafil. Values decreased with reduced temperature. At 28°C the inhibition-ratios for sildenafil was 0.029, and 0.294 for vardenafil (**Table 7**).

DISCUSSION

Temperature reduction does not affect the ability of PDE5-inhibitors sildenafil and vardenafil to reach their cytosolic site of action. Our results show that both drugs inhibit

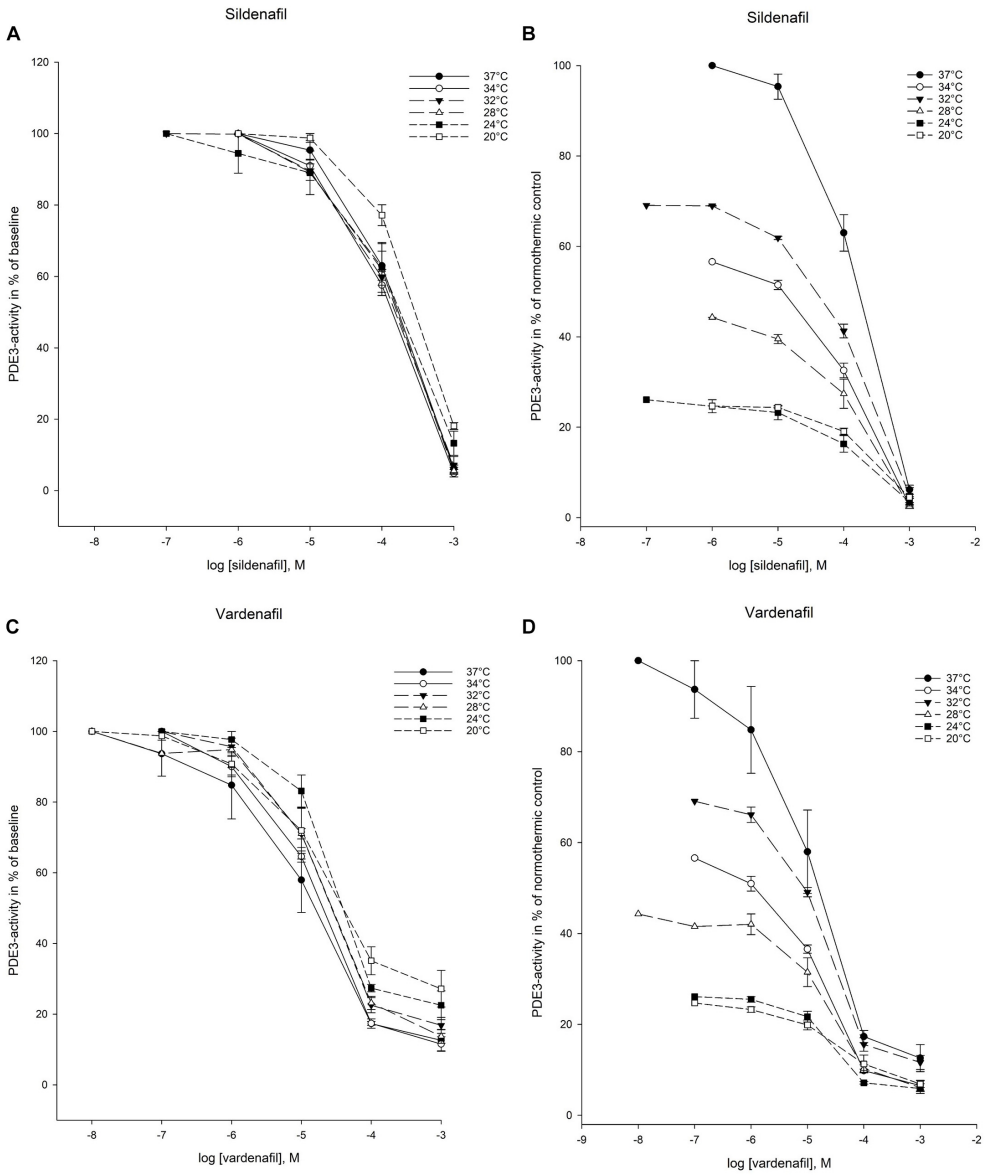

FIGURE 4 | Temperature-dependent inhibition of phosphodiesterase-3 (PDE3) by vardenafil and sildenafil. **(A)** Sildenafil inhibition curves for PDE3-activity at temperatures ranging from 37°C to 20°C. The doses of sildenafil are in logarithm of the concentration in mol/L. **(B)** Inhibition curves for PDE3-activity by sildenafil in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. The doses of sildenafil are in logarithm of the concentration in mol/L. **(C)** Vardenafil inhibition curves for PDE3-activity at temperatures ranging from 37°C to 20°C. The doses of vardenafil are in logarithm of the concentration in mol/L. **(D)** Inhibition curves for PDE3-activity by vardenafil in % of normothermic inhibition curve at temperatures ranging from 37°C to 20°C. The doses of vardenafil are in logarithm of the concentration in mol/L.

elimination of cGMP, as well as cAMP, at temperatures down to 20°C. The concentration of sildenafil needed to inhibit PDE-enzymes is increased with temperature reduction, while there is a tendency toward increased sensitivity of efflux pumps to sildenafil-inhibition. Vardenafil concentrations needed to inhibit all elimination pathways remain unchanged during temperature reduction. Establishing the pharmacodynamic properties of these PDE5-inhibitors is crucial in the process of developing better treatment-guidelines for cardiovascular complications in accidental and therapeutic hypothermia.

Accidental hypothermia guidelines recommend to avoid pharmacological treatment until a body core temperature of minimum 30°C is reached (No Author, 2000; Truhlar et al., 2015). Subsequently, use of vasopressors are listed as the preferred drugs for cardiovascular support, despite the benefits being unclear (Paal et al., 2016). There are indications that this strategy might be unfavorable. In experimental hypothermia and rewarming, increased afterload is associated with HCD and a poor outcome (Dietrichs et al., 2014a, 2015; Håheim et al., 2017). Contradictory to current recommendations, pharmacologically induced vasodilation emerge as a promising strategy to prevent

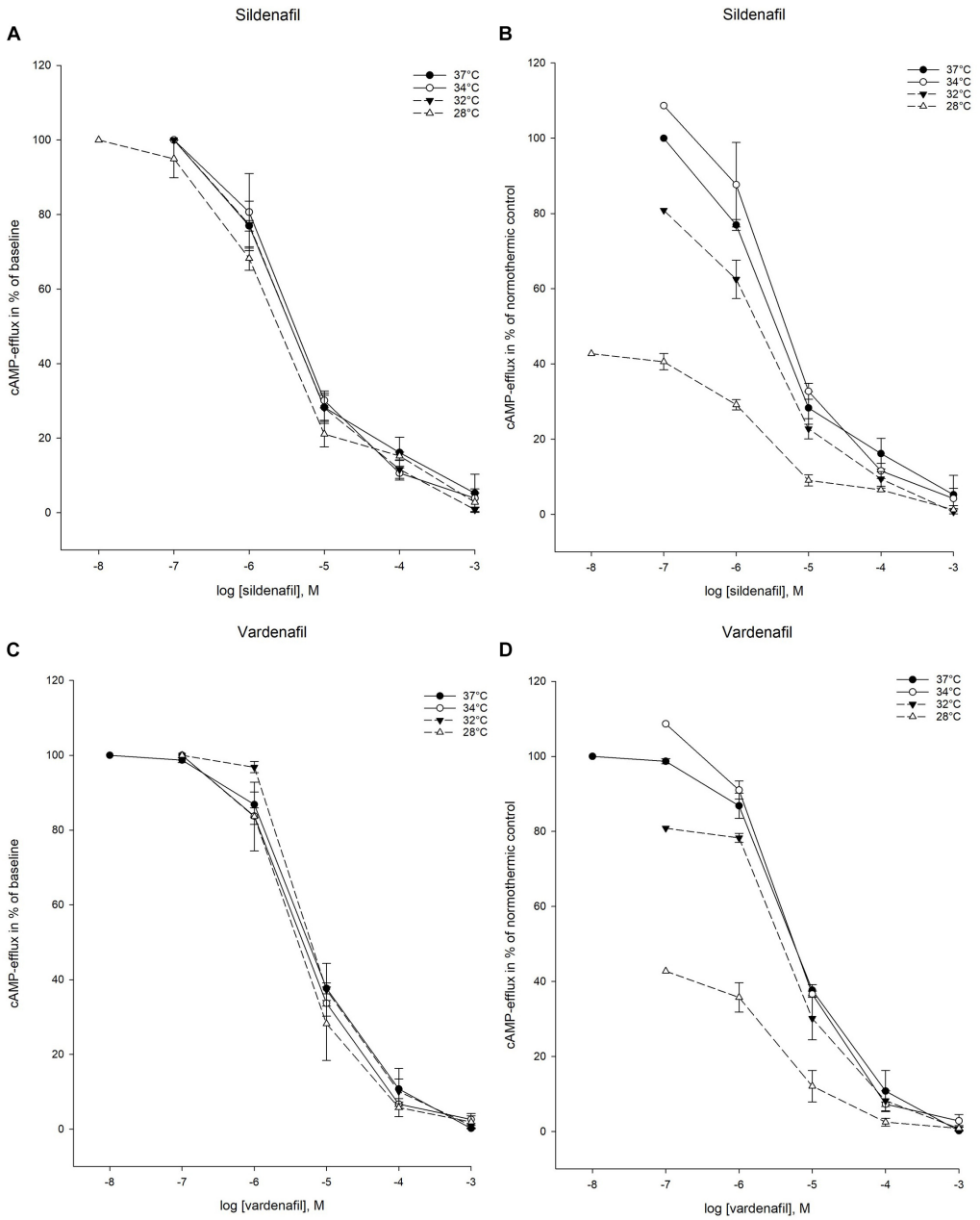

FIGURE 5 | Temperature-dependent inhibition of cAMP-efflux in inside-out vesicles (IOV). **(A)** Sildenafil inhibition curves for ABCC4-activity at temperatures ranging from 37°C to 28°C. The doses of sildenafil are in logarithm of the concentration in mol/L. **(B)** Inhibition curves for ABCC4-activity by sildenafil in % of normothermic inhibition curve at temperatures ranging from 37°C to 28°C. The doses of sildenafil are in logarithm of the concentration in mol/L. **(C)** Vardenafil inhibition curves for ABCC4-activity at temperatures ranging from 37°C to 28°C. The doses of vardenafil are in logarithm of the concentration in mol/L. **(D)** Inhibition curves for ABCC4-activity by vardenafil in % of normothermic inhibition curve at temperatures ranging from 37°C to 28°C. The doses of vardenafil are in logarithm of the concentration in mol/L.

HCD and elevate organ blood flood during rewarming (Håheim et al., 2017, 2020). cGMP elevation is a central mechanism for vasodilation that remains intact during severe hypothermia and rewarming (Håheim et al., 2017, 2020). Pharmacological cGMP-increase is achieved either through stimulating intracellular production, or through reducing elimination. At low core temperatures, it is apparent that drug-induced cGMP production, through stimulating the NO-GC-cGMP-PKG-pathway, is a challenging strategy. Doses of nitroprusside giving favorable effects in normothermia proved harmful in severe hypothermia, due to elevated potency, while doses adjusted to effect on mean arterial pressure (MAP) reduction were favorable (Håheim et al., 2017). In this context, elevating intracellular cGMP through inhibiting elimination, could prove a physiological approach

TABLE 7 | Drug selectivity for sildenafil and vardenafil at temperatures ranging from 37°C to 20°C.

Drug selectivity [IC_{50}]/[IC_{50}]	[PDE3-inhibition]/[PDE5-inhibition]		[cAMP-efflux-inhibition]/[cGMP-efflux-inhibition]		[cGMP-efflux-inhibition]/[PDE5-inhibition]		[cAMP-efflux-inhibition]/[PDE3-inhibition]	
Temperature	**Sildenafil**	**Vardenafil**	**Sildenafil**	**Vardenafil**	**Sildenafil**	**Vardenafil**	**Sildenafil**	**Vardenafil**
37°C	14400	1960	0.832	1.11	815	625	0.047	0.354
34°C	6730	1650	1.61	0.620	269	622	0.064	0.234
32°C	4240	2630	1.84	1.99	105	422	0.046	0.320
28°C	5780	2220	0.917	1.95	180	335	0.029	0.294
24°C	5110	2510	–	–	79.7	170	–	–
20°C	7270	3230	–	–	74.5	480	–	–

Values are ratios between IC_{50}-values for different elimination ways of cAMP and cGMP. Neither of the drugs inhibited cAMP-efflux below 28°C.

to achieve reduction of afterload, without causing uncontrolled reduction of MAP.

Our findings demonstrate that the difference between IC_{50}-values for cGMP-efflux-inhibition and PDE5-inhibition decreases during cooling, indicating that the elimination of cGMP during hypothermia is more dependent on cGMP-efflux. IC_{25}–IC_{75} intervals were calculated to estimate a pharmacodynamic window of effect for both drugs on all elimination pathways. No decrease was detected during hypothermia. Targeting cGMP-efflux, in addition to PDE5, therefore appears to be a relevant strategy for cardiovascular support during rewarming from hypothermia. We show that sildenafil and vardenafil are both able to inhibit these cGMP-elimination pathways at low core temperatures, and that they therefore show potential for treatment of hypothermic patients.

There is consensus of venoarterial extra corporeal membrane oxygenation (VA-ECMO) being the preferred treatment of hemodynamically unstable hypothermic patients (No Author, 2000; Brown et al., 2012; Truhlar et al., 2015; Saczkowski et al., 2018). During such treatment, cardiovascular complications are common (Rao et al., 2018; Choi et al., 2019). Left ventricle (LV) dysfunction can appear as not all of the circulating blood is directed through the VA-ECMO device. Some will still pass through the pulmonary circulation. Since MAP is increased by VA-ECMO, the LV has to overcome increased afterload to maintain ejection fraction. Failure could lead to LV-distention and elevated pressure, with a backward failure giving increased pulmonary pressure and edema (Lo Coco et al., 2018; Rao et al., 2018). Risk is higher if the patient has an underlying LV-dysfunction, like HCD (Dietrichs et al., 2018). Pharmacological afterload reduction is a suggested treatment strategy to alleviate backward failure during VA-ECMO-treatment, but it is important to avoid systemic hypotension (Rao et al., 2018). In the present experiment, inhibition of cGMP-efflux is achieved at supratherapeutic concentrations during both normothermia and hypothermia, while PDE5 inhibition by sildenafil or vardenafil, appears a promising strategy to achieve physiologically balanced afterload reduction, and prevent pulmonary edema in hypothermic VA-ECMO patients (Lo Coco et al., 2018).

In addition to afterload reduction, inotropic support could also be favorable during VA-ECMO-treatment (Rao et al., 2018). Inotropes are administered to help overcome the increased afterload and maintain LV ejection fraction (Rao et al., 2018). We show that sildenafil and vardenafil inhibit elimination of cAMP during hypothermia. Earlier studies have shown positive inotropic effect of PDE3-inhibition *in vivo* (Dietrichs et al., 2014a, 2018). IC_{50}-values for PDE5-inhibition by sildenafil and vardenafil are in the nM-range, while IC_{50}-values for PDE3 and inhibition of cAMP-efflux are in the μM-range. These concentrations are supratherapeutic during normothermia (**Tables 4, 5**). In order to provide evidence-based inotropic support during VA-ECMO-treatment in hypothermic patients, further studies on drugs that target inhibition of cAMP-elimination are needed as sildenafil and vardenafil appears to be ineffective in therapeutic doses.

Treatment of hemodynamically unstable hypothermic patients, face some of the same challenges as pre-hospital HAPE-treatment, when evacuation is difficult, and the patient is exposed to low temperatures. Sildenafil has been proposed as treatment, as it can be administered when oxygen treatment and rapid decent is impossible (Bates et al., 2007). The strong linear correlation between IC_{50}-value and decreasing temperature may serve as a helpful tool in low ambient temperatures, as effect is decided according to measured core temperature. Further investigation of pharmacokinetic data could complement this finding and help develop evidence-based guidelines, with pinpointed dose recommendations for cardiovascular support in hypothermic patients. Although sildenafil and tadalafil are the only PDE5-inhibitors suggested in the treatment of HAPE (Maggiorini et al., 2006; Bates et al., 2007), vardenafil may now be suggested as a candidate drug. Our findings show little pharmacodynamic change, meaning that clinicians only need to account for the impact of hypothermia on pharmacokinetic properties, when calculating adequate vardenafil dosage.

Metabolism of sildenafil and vardenafil is performed in the liver mainly by CYP3A4 but also CYP2C9 and CYP3A5 (Huang and Lie, 2013). Enzyme affinity is decreased with reduction of core temperature, impeding elimination (Tortorici et al., 2006; van den Broek et al., 2010). CYP3A4, the main metabolizing enzyme of both sildenafil and vardenafil is shown to have an activity of 48% at 26°C and 68% at 32°C (Fritz et al., 2005). Sildenafil and vardenafil are both metabolized to active metabolites that are less potent

than the parent compounds (Hyland et al., 2001; Bischoff, 2004b). These metabolites are largely eliminated through biliary excretion (Mehrotra et al., 2007), which also is impaired during hypothermia (van den Broek et al., 2010). Decreased activity of CYP-enzymes and biliary excretion during hypothermia, alongside changed plasma protein binding (van den Broek et al., 2010), will lead to slower metabolism of the PDE5-inhibitors, slower production of active metabolites, reduced excretion, altered free fraction and thus, unpredictable therapeutic effect and increased risk of toxicity. In order to safely introduce the use of PDE5-inhibitors in treatment of cardiovascular complications during hypothermia and rewarming, these pharmacokinetic aspects need to be addressed through further experiments.

Although PDE3 and PDE5 are the main targets for pharmacological agents aiming to treat cardiovascular conditions through PDE-inhibition, other PDEs could also be affected by sildenafil and vardenafil administration. None have been investigated during hypothermia. In therapeutic concentrations of the PDE5-inhibitors, the isoenzyme closest in IC_{50}-value, and of cardiovascular relevance, is PDE1 (Bischoff, 2004a; Levy et al., 2011). PDE2, PDE4, PDE6 and PDE9 may also influence cardiovascular functions by inhibition of metabolism of cAMP, cGMP or both. However, these PDEs have much higher IC_{50}-values for sildenafil and vardenafil during normothermia (Adderley et al., 2010; Kim and Kass, 2017). Studies on other PDEs were excluded from our experiment due to PDE5 being the main target for afterload reduction by vascular smooth muscle and PDE3 being the target for inotropic support in cardiac muscle. Our results show that sildenafil and vardenafil largely remain specific for PDE5. Further studies looking at other relevant PDEs in our hypothermic model would provide a better overall description on possible inhibition of other PDEs by sildenafil and vardenafil. PDEs are also known to interact when present in the same tissue or experimental solutions (Zhao et al., 2015). Assessing these different aspects could provide more information about potential effects and side effects of the drugs during hypothermia and therefore remains to be studied in future studies in our model.

CONCLUSION

Sildenafil and vardenafil are able to reach cytosol and IC_{50}-values for cGMP-elimination remain intact or predictable at temperatures down to 20°C. As the cellular effects of these drugs can cause afterload reduction, they show potential in treating cardiovascular dysfunction during hypothermia. Our findings lay foundation for *in vivo* studies and further development of evidence-based, pharmacological treatment guidelines in both accidental and therapeutic hypothermia, as well as in HAPE-patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, AK, NS, and TK conducted the experiments in the lab. RL helped with technical issues and theoretical questions during experiments. O-MF analyzed the results using mass-spectrometry. AS and ED interpreted the results and performed the statistics. AR, TT, GS, and ED planned the research project. AS, ED, O-MF, NS, and GS contributed to the manuscript. All authors read and approved the manuscript.

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