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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

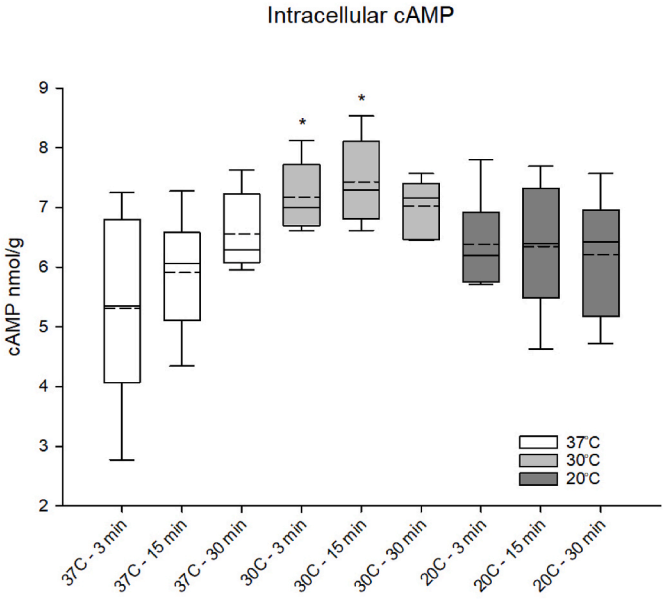

Fig. 1. Intracellular levels of cAMP after 3, 15 or 30 min of incubation in temperatures 37 °C, 30 °C, and 20 °C. The concentrations are presented as means ± SEM and given in nmol/g. * Significant difference (P-value <0.05), when compared to normothermic control.

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 *

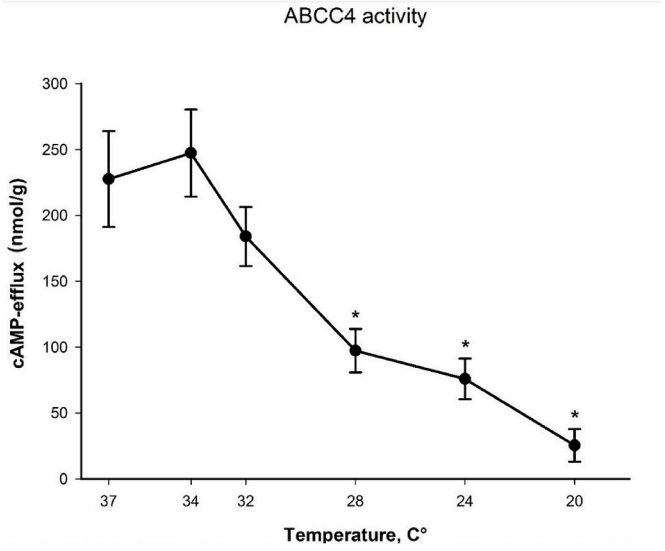

Fig. 2. Temperature dependent effects on cellular efflux of cAMP. The concentrations are presented as means ± SEM and given in nmol/g. * Significant difference (P-value <0.05), when compared to normothermic control.

(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

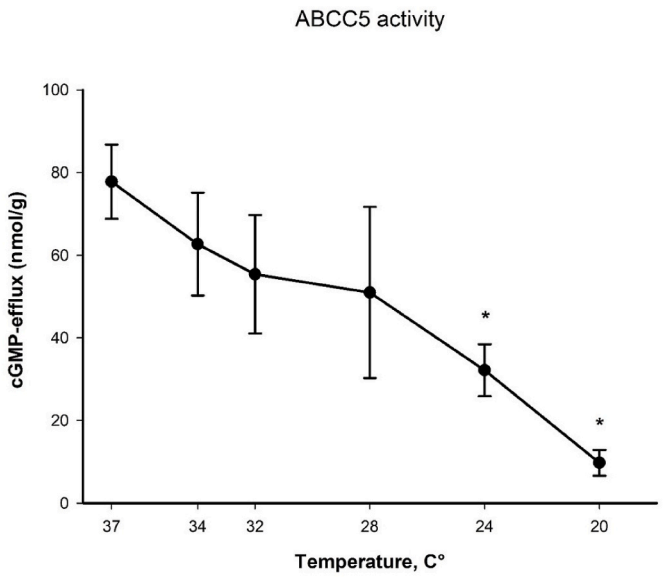

Fig. 3. Temperature dependent effects on cellular efflux of cGMP. The concentrations are presented as means ± SEM and given in nmol/g. * Significant difference (P-value <0.05), when compared to normothermic control.

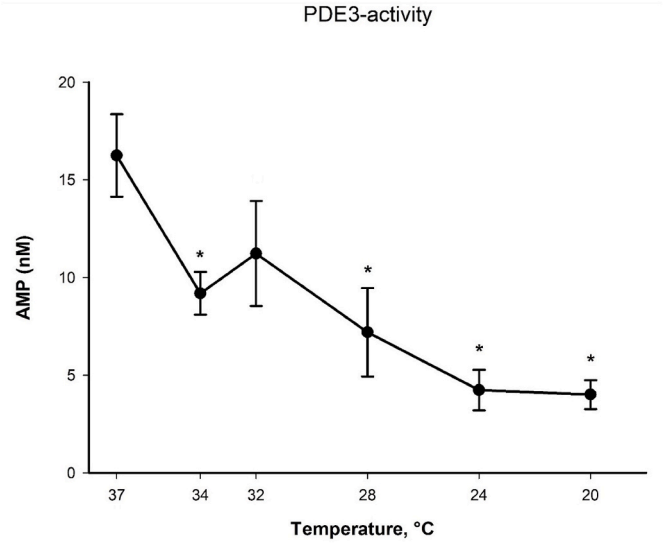

Fig. 4. Temperature dependent effects on PDE3 activity. The concentrations are presented as means ± SEM and given in nM. * Significant difference (P-value <0.05), when compared to normothermic control.

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

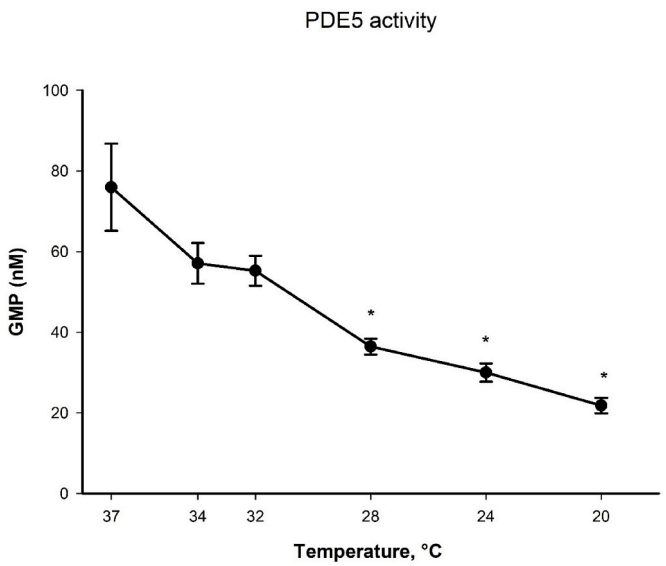

Fig. 5. Temperature dependent effect on PDE5 activity. The concentrations are presented as means ± SEM and given in nM. * Significant difference (P-value <0.05), when compared to normothermic control.

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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