SGLT2 independent effects of the SGLT2 inhibitor empagliflozin

Focus on the heart

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Chapter 04

Protease XIV abolishes NHE1 inhibition by empagliflozin in cardiac cells

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Abstract

Background: SGLT2i directly inhibit the cardiac sodium-hydrogen exchanger-1 (NHE1) in isolated ventricular cardiomyocytes (CMs). However, other studies with SGLT2i have yielded conflicting results. This may be explained by methodological factors including cell isolation techniques, cell types and ambient pH. In this study, we tested whether the use of protease XIV (PXIV) may abrogate inhibition of SGLT2i on cardiac NHE1 activity in isolated rabbit CMs or rat cardiomyoblast cells (H9c2), in a pH dependent manner.

Methods: Rabbit ventricular CMs were enzymatically isolated from Langendorff-perfused hearts during a 30-min perfusion period followed by a 25-minute after-dissociation period, using a collagenase mixture without or with a low dose PXIV (0.009 mg/ml) present for different periods. Empagliflozin (EMPA) inhibition on NHE1 activity was then assessed at pH of 7.0, 7.2 and 7.4. In addition, effects of 10 minutes PXIV treatment were also evaluated in H9c2 cells for EMPA and cariporide NHE1 inhibition.

Results: EMPA reduced NHE1 activity in rabbit CMs that were not exposed to PXIV treatment or undergoing a 35-minutes PXIV treatment, independent of pH levels. However, when exposure time to PXIV was extended to 55 minutes, NHE1 inhibition by EMPA was completely abolished at all three pH levels. In H9c2 cells, NHE1 inhibition by EMPA was evident in non-treated cells but lost after 10-minutes incubation with PXIV. NHE1 inhibition by cariporide was unaffected by PXIV.

Conclusion: The use of protease XIV in cardiac cell isolation procedures obliterates the inhibitory effects of SGLT2i on NHE1 activity in isolated cardiac cells, independent of pH.

Introduction

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) have revolutionized the medical treatment of diabetic and heart failure patients[1]. Large clinical trials have demonstrated a significant decrease of approximately 30% in hospitalization for heart failure, and meta-analysis of different SGLT2i trials has also reported a substantial reduction in mortality[2]. SGLT2i medications were originally developed to target the SGLT2 protein in the kidney, where it facilitates glucose reabsorption from the kidney filtrate into the blood. Inhibition of SGLT2 leads to glycosuria, resulting in the excretion of 40-75 grams of glucose per day in diabetic or prediabetic patients[3]. Apart from their SGLT2-dependent effects on the kidney, including modulating of substrate metabolism, fluid balance, and hemodynamics, SGLT2i has been found to have direct effects on various cell types largely lacking SGLT2, such as cardiomyocytes, endothelial cells, platelets and fibroblast[4,5]. These effects also involve myocardial infarction and ischemia-reperfusion (I/R)[6]. Indeed, it was shown that empagliflozin (EMPA) protection against infarct development in I/R was independent of the presence of SGLT2[7]. The sodium-hydrogen exchanger-1 (NHE1) was the first off-target cardiac mechanism of SGLT2i to be discovered[8,9]. It has been demonstrated that SGLT2i inhibits NHE1 activity in isolated cardiomyocytes from rabbits and mice, leading to a decrease in intracellular Na^+ and Ca^{2+} and an increase in mitochondrial calcium. While the inhibition of NHE1 by SGLT2i in isolated cardiomyocytes has been replicated in several studies involving different cell types[10-16], other studies have reported conflicting results[17]. In a previous investigation, we examined various experimental factors that differed among these conflicting studies, which could potentially account for the discrepancies.

It has been reported that enzymes used in cell isolation procedures can harm channel proteins embedded in the plasma membrane. For instance, protease XIV and XXIV, which are specific serine proteases, were found to degrade hERG1 potassium current and channel in HEK cells and cardiomyocytes[18]. Consequently, the choice of cell isolation procedure can significantly impact the ion channels and exchangers within the plasma membrane. This raises the possibility that the loss of NHE1 inhibition by SGLT2i may be attributed to the use of specific enzymes in the cell isolation procedure.

We also questioned whether the PXIV effects on NHE1 by EMPA was specific for freshly isolated rabbit cardiac cells or more a general phenomenon applicable to other cardiac cells as well. We therefore also used another cardiac cell type. Therefore, in this study, we aimed to investigate whether the exposure time of serine protease XIV disrupts inhibition of NHE1 by SGLT2i in freshly isolated rabbit cardiac cells. We next tested whether 2) this effect was dependent on pH, 3) was also present in cardiac precursor cells, and 4) iNHE inhibition by the classic NHE inhibitor cariporide was also affected by PXIV. We conclude that inhibition of the NHE by EMPA is strongly

dependent on duration of protease incubation both in rabbit cardiomyocytes and rat cardiac precursor cells, and that it is independent on pH.

Methods

Preparation of isolated ventricular cardiomyocytes by enzymatic dissociation

The study was approved by the local animal experiments committee of the Academic Medical Center, Amsterdam, The Netherlands. Rabbits were housed for at least one week, with a 12 h day/night cycle, and food and drinking water ad libitum. Male New Zealand White rabbits (2.5-3.5 kg) were anaesthetized with 35mg/kg ketamine and 10mg/kg xylazine subcutaneously and heparinized with a bolus of 1000 IU heparin (intravenously). Subsequently, the animals were euthanized by pentobarbital 50 mg/kg intravenously, the thorax was opened and the heart was quickly removed and submerged in ice-cold perfusion solution (composition see below). After cannulation of the aorta, the heart was mounted on a Langendorff perfusion setup, and perfused for 15 min at constant pressure (50 mm Hg) at 37 °C with a modified Tyrode's solution containing (in mM) 128 NaCl, 4.7 KCl, 1.45 $CaCl_2$, 0.6 $MgCl_2$, 27 $NaHCO_3$, 0.4 NaH_2PO_4 and 11 glucose (pH maintained at 7.4 by equilibration with a mixture of 95% O_2 and 5% CO_2) to remove remaining blood from the heart. Next, perfusion was changed to a nominally calcium-free solution containing (in mM) 16.8 HEPES, 146.4 NaCl, 3.3 $KHCO_3$, 1.4 KH_2PO_4, 1.0 $NaHCO_3$, 2.0 $MgCl_2$, 0.01 $CaCl_2$, 11.0 glucose, pH 7.3 (NaOH). After 15 min, collagenase type B (0.15 mg/ml, Roche 11088815), collagenase type P (0.05 mg/ml, Roche 11213865), trypsine inhibitor (0.1 mg/ml, Roche 10109878), 0.2 mg/ml hyaluronidase (Sigma H-3506), and creatine (10 mM) were added, and the heart was perfused for another 30 min at a constant flow in a recirculating manner. During the final 30-minute period, protease XIV (Sigma H-3506) was either absent, present only during the last 10 minutes of perfusion, or present throughout the entire 30-minute perfusion. Subsequently, the left ventricular wall was further dissociated using the following after-dissociation protocol: the tissue was cut into small pieces, incubated in the enzyme-containing nominally calcium-free solution with or without PXIV (see Fig. 1), and gently agitated using a gyrotory water bath shaker for 25 minutes. During the last 10 minutes, 1% albumin (fatty acid free, Roche 10775835001) was added to the enzyme-containing dissociation solution. All dissociation solutions were saturated with 100% O_2 and the temperature was maintained at 37 °C. Cells were allowed to sediment and were resuspended in enzyme-free dissociation solution supplemented with 1% albumin and 1.3 mM $CaCl_2$. Only rod-shaped myocytes with clear striations were selected for NHE1 activity measurement.

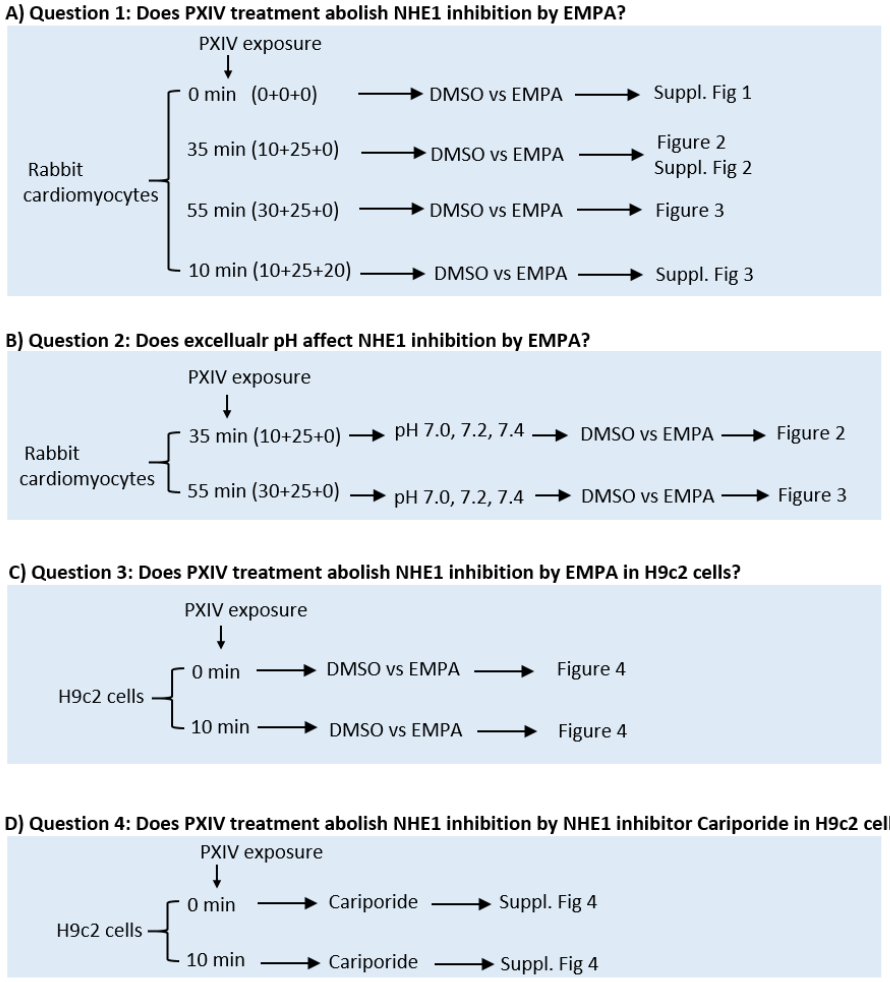


Figure 1 Overview of the four research questions addressed to study effects of various durations of Proteinase XIV exposure and extracellular pH's for determination of NHE1 activity in isolated rabbit cardiomyocytes and H9c2 cells. A, B: Rabbit cardiomyocytes are isolated on a Langendorff set-up by perfusing with a fixed mixture of collagenases with or without different treatment periods of proteinase XIV. Next, rabbit cardiomyocytes are incubated with the pH fluorescent probe SNARF-AM in a water bath for 20 min in absence or presence of PXIV. NHE1 activity in cardiomyocytes is measured by fluorescence microscopy where different pH conditions of HEPES solution treated with or without EMPA are applied during the measurement. C, D: H9c2 cells were treated with DMSO, EMPA or NHE1 inhibitor Cariporide with or without PXIV during the NH_4^+-treatment and NHE1 activity measurements. EMPA: Empagliflozin. Fig 1A for question 1 and 2, exposure time of

PXIV is given as the sum of (X+Y+Z), whereby X= PXIV exposure during cell dissociation on Langendorff system, Y= PXIV exposure during after-dissociation in gyrotory water bath shaker (cells are taken out from gyrotory every 5 min), Z= PXIV exposure during SNARF-AM Incubation in waterbath. For questions 3 and 4, PXIV exposure is during the 10 min NH_4^+-treatment.

Cell culture

H9c2 rat cardiomyoblast cells were obtained from ATCC (American Type Culture Collection) and cultured in high glucose (4,500 mg/l) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and 100 U/ml of Penicillin/Streptomycin. In brief, H9c2 cells were digested with trypsin (0.25%), recovered by centrifugation (5 min, 1000 rpm) and re-suspended in plating medium. Cells were grown in an atmosphere of 95% O_2, 5% CO_2 in a humidified incubator. Stock cultures were passaged at 2- to 3-day intervals. 50.000 cells were seeded and grown on coverslips for NHE1 activity measurement.

NHE1 activity measurement

To measure intracellular pH, isolated rabbit cardiomyocytes and H9c2 cells were incubated with 10 µM SNARF-AM (Thermofisher, C1270) for 30 min in a 37 °C water bath (Fig. 1A) in HEPES solution (mM): HEPES 17.0, $NaHCO_3$ 1.0, $KHCO_3$ 3.3, KH_2PO_4 1.4, $CaCl_2$ 1.3, $MgCl_2$ 2.0, NaCl 144, pH adjusted with NaOH to 7.0 or 7.2 or 7.4. In a separate set of experiments, isolated rabbit cardiomyocytes which had been exposed to 0.009 mg/ml Protease XIV for 35 minutes during enzymatic dissociation protocol (Figure 1), were re- incubated with 0.009 mg/ml Protease XIV for an additional 20 min during SNARF-AM incubation. SNARF-loaded rabbit cardiomyocytes were attached to a poly-D-lysine (0.1 g/l) treated coverslip for NHE1 activity measurement. The general workflow of the procedures described above and the research questions addressed is depicted by a schematic drawing in Figure 1A and 1B. Experiments were conducted using a temperature controlled (37 °C) perfusion chamber (height 0.4 mm, diameter 10 mm, volume 30 µl) in which the coverslips were placed. NHE1 activity was measured by recording SNARF-fluorescence (580/640 nm emission; 515 nm excitation) following a NH_4^+ pulse[8,14]. Shortly, after 30 sec stabilization, medium was quickly replaced (< 1 sec) with the same solution now containing 20 mM NH_4Cl for 10 min, resulting in intracellular alkalosis. After 10 min, the NH_4Cl was quickly replaced (< 1 sec) with normal solution, which resulted in an almost instantaneously intracellular acidosis, followed by the recovery from acidosis, which was monitored for the next 5 min. The effect of 1 µM EMPA (MedChem Express, Monmouth Junction, NJ, USA) or 0.02% DMSO (Sigma/Merck, D2650) was tested on recovery of acidosis and these compounds were continuously present during the NH_4Cl pulse and the washout period of NH_4Cl. Additionally, H9c2 cells, were subjected or not to 0.009 mg/ml Protease XIV during the 10 min NH_4Cl pulse and the washout period of NH_4Cl during which either DMSO, EMPA or cariporide (10 µM) were also present, to address the questions

whether NHE inhibition by EMPA or the classic NHE inhibitor cariporide was dependent on the presence of PXIV in this different cell type.(Figure 1C and D). The initial rate of intracellular H^+ recovery following washout of ammonium is used as estimate of NHE1 activity. This initial rate equaled the slope of the linear fit of the intracellular H^+ during the first 50 sec of H^+ recovery.

Statistics

Statistical analysis was performed using GraphPad Prism v9. Results are presented as mean ± SEM for normally distributed data or median ± IQ for non-normally distributed data. Data normality was examined using the Shapiro-Wilk test (with α=0.05). For normally distributed data, the Paired t test (unpaired 2-tailed) was used for 2-group analysis. For non-normally distributed data, the Mann-Whitney test was used for 2-group analysis, and the Kruskal-Wallis test with Dunn's multiple comparisons test was used for multiple-group analysis. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Results

We first established the presence of NHE1 inhibition by EMPA with our standard cell isolation procedure without PXIV treatment. Typical examples from rabbit ventricular CMs clearly demonstrate the failure to recover from intracellular acidosis in the presence of EMPA compared to DMSO (Suppl. Fig. 1A). NHE1 activity was calculated from the slope of the linear fit of the first 50 sec of $[H^+]$ recovery after $[H^+]$ had reached its maximum value (Suppl. Fig. 1B). At an extracellular pH of 7.4, EMPA significantly altered the negative slope of the linear fitted $[H^+]$ recovery of DMSO-treated cells towards a more positive value, indicating decreased recovery of $[H^+]$ due to NHE 1 inhibition by EMPA (Suppl. Fig. 1C).

We next investigated whether a 35 minutes exposure toPXIV (0.009 mg/ml) during the dissociation protocol affected NHE1 inhibition by EMPA, and whether this inhibition was dependent on the extracellular pH. The mean pH- traces during and after the NH_4^+ treatment for DMSO- and EMPA-treated CMs are shown in Fig.2A, demonstrating strong inhibition of pH recovery following NH_4^+ withdrawal in the presence of EMPA as compared to DMSO only. The individual pH traces per animal for each of the 7 rabbits are given by supplementary Figure 2. Following the conversion of these mean pH tracings into mean changes of $[H^+]$ (Fig. 2B), the slope of the linear fit of $[H^+]$ recovery approximates zero (0.06 ± 0,57) in the presence of EMPA, whereas the slope of the linear fir is -1.44 (-1,44 ± 1,23, $p<0.001$) in the presence of DMSO, indicative of inhibition of $[H^+]$ recovery by EMPA at pH 7.4 . The individual and average slope values for 35 minutes PXIV at the different extracellular pH's, are depicted in Fig. 2C-2E. At pH 7.0, 7.2 and 7.4 EMPA significantly reduced NHE1 activity as compared to DMSO treated rabbit cardiomyocytes (Fig. 2C). This indicates that short term PXIV treatment or changes in extracellular pH in the range of 7.0-7.4, leave NHE1 inhibition by EMPA intact.

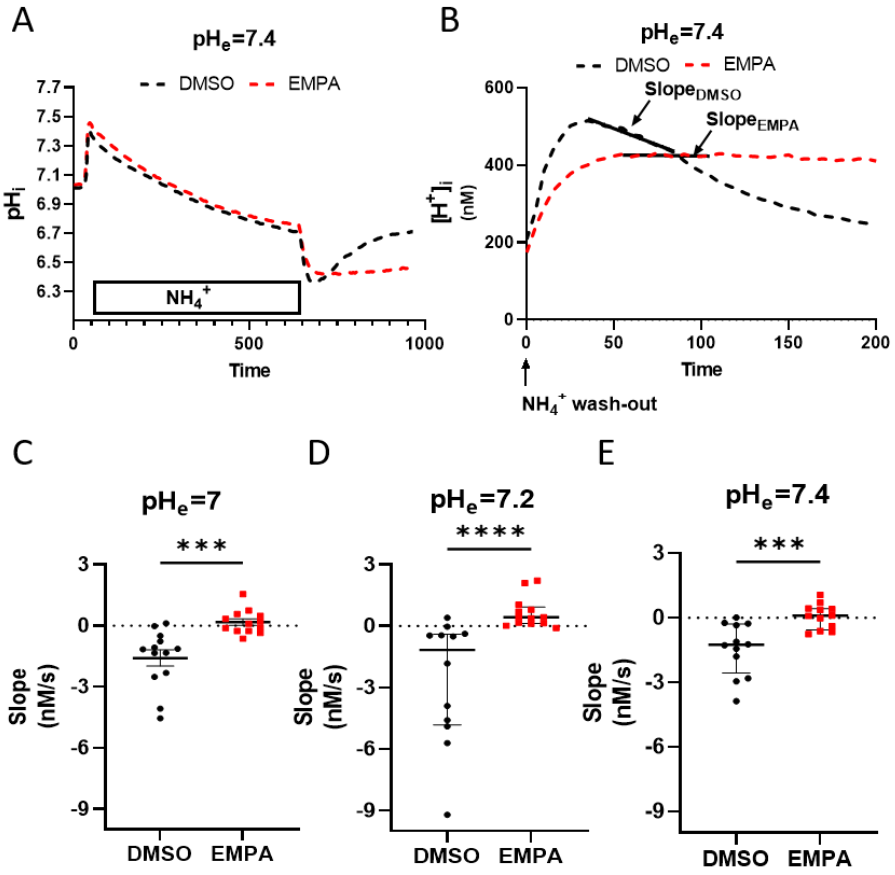


Figure 2 Empagliflozin inhibits NHE1 activity in rabbit CMs exposed to PXIV for 35 minutes during enzymatic dissociation. (A) Average curve changes of intracellular pH in response to a NH_4^+ pulse for DMSO- and EMPA-treated CMs; (B) Average curve changes of intracellular $[H^+]$ during the first 200 sec after NH_4^+ wash-out, showing the slope; (C-E) NHE1 activity indicated by the slope of the linearly fitted $[H^+]$ recovery for DMSO- and EMPA-treated cell at different extracellular pH's (pH=7.0: n=13/13 cells from 7 rabbits, Paired t test (two tailed); pH=7.2: n=12 (DMSO)/13(EMPA) cells from 7 rabbits, Mann-Whitney test; pH=7.4: n=12/12 cells from 7 rabbits, Paired t test (two tailed)). EMPA: Empagliflozin. Slope ($\Delta[H^+]/\Delta s$) of the linear fit of the first 50 s of intracellular $[H^+]$ recovery. *** $P < 0.001$, **** $P < 0.0001$.

We then investigated whether prolonged exposure to PXIV (55 minutes) during the enzymatic dissociation protocol affects NHE1 inhibition by EMPA or influences pH effects on inhibition (Fig. 3). The mean pH tracings following 55 minutes PXIV (Fig. 3A), now show pH recovery to occur under both DMSO and EMPA

conditions. Accordingly, the slope of the linear fit of the mean [H^+] tracing for EMPA shows a significant negative slope, similar to the DMSO condition, indicative of pH recovery (Fig. 3B). The absent of an EMPA-effect was consistent at extracellular pH 7.0, 7.2 and 7.4. Thus, the increased exposure time of PXIV during the dissociation procedure, abolished the effects on NHE1 activity by EMPA, independent of the extracellular pH levels (Fig. 3C). Next, we determined whether PXIV can also abrogate EMPA-NHE1 inhibition when prolonged exposure to PXIV is applied after the cell isolation procedure. CMs that were exposed for 35 minutes to PXIV during the enzymatic dissociation procedure, were subsequently incubated for another 20 minutes with PXIV during SNARF-incubation. Supplementary figures 3A and 3B are examples of pH and [H^+] changes in response to an NH_4^+ pulse and subsequent NH_4^+ washout in cells treated solely for 35 minutes with PXIV (extracellular pH of 7.2), showing a clear inhibitory effect of EMPA on NHE1 activity (Suppl. Fig. 3C). On the other hand, CMs that were exposed to an additional 20 minutes of PXIV, EMPA was not effective in blocking NHE1 activity (Suppl. Fig. 3D-F).

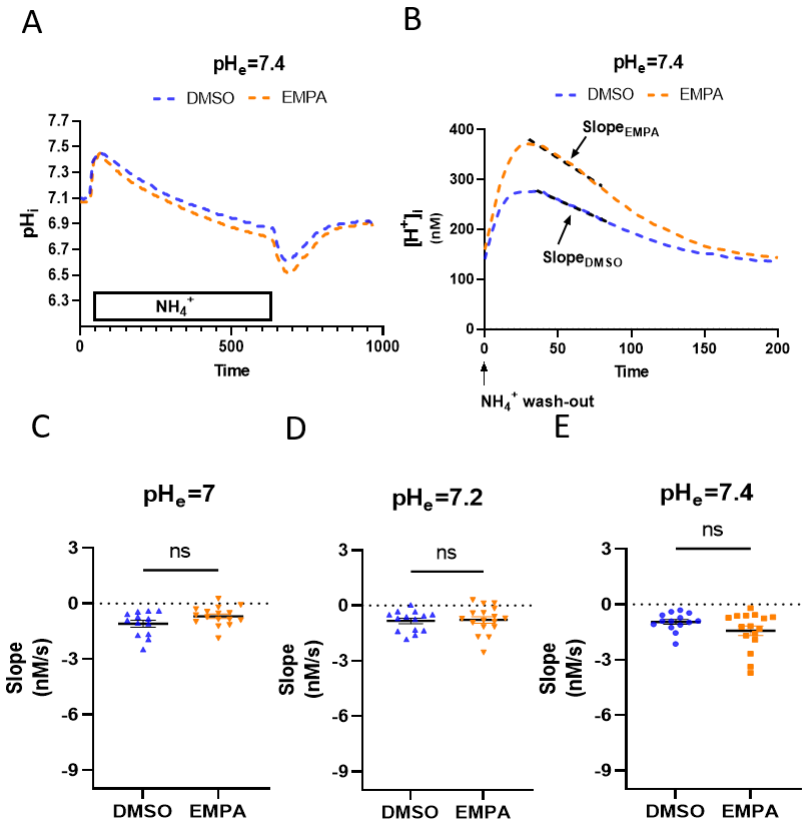
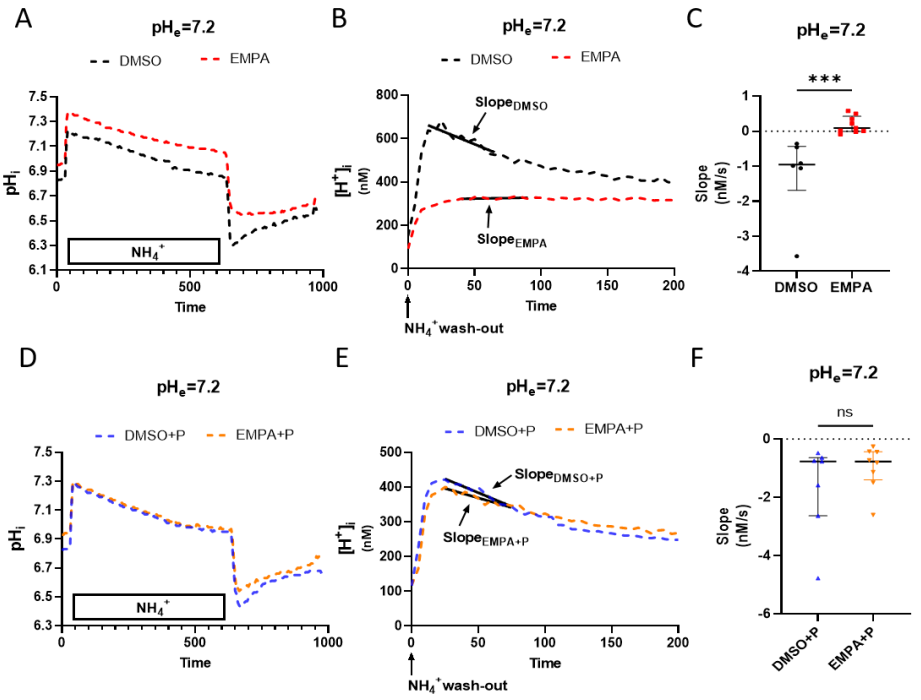


Figure 3 Empagliflozin does not inhibit NHE1 activity in rabbit CMs exposed to PXIV for 55 minutes during enzymatic dissociation. Average curve changes of intracellular pH for DMSO and EMPA treatment; (B) Average curve changes of intracellular [H^+] during the first 200 sec after NH_4^+ wash-out, showing the slope for DMSO and EMPA. (C-E) NHE1 activity as reflected by the slope for DMSO- and EMPA-treated cell at different extracellular pH's (pH=7.0: n=13/16 cells from 5 rabbits, Paired t test (two tailed); pH=7.2: n=14(DMSO)/17(EMPA) cells from 5 rabbits, Paired t test (two tailed); pH=7.4: n=14/16 cells from 6 rabbits, Paired t test (two tailed)). EMPA: Empagliflozin. Slope ($\Delta[H^+]/\Delta s$) of the linear fit over first 50 s of intracellular [H^+] recovery. ns $P > 0.05$.

To study whether the PXIV effects also extend to other cell types, we employed the rat cultured myoblast cell line H9c2. EMPA effects on NHE1 activity at pH 7.2 were determined in the absence or presence of 0.009 mg/ml PXIV, administered simultaneously with the 10 min NH_4^+ treatment (Figure 4). Figure 4A and B show that EMPA also in this cardiac cell type inhibits the NHE1, as reflected by the mean course of changes in intracellular pH and H^+ following the NH_4^+ pulse in the presence of EMPA (compared to DMSO-treatment), and the accompanying averaged and individual slope values (Figure 4C). However, when cells were treated with PXIV during the NH_4^+ pulse and the subsequent pH recovery period, recovery of pH (Fig. 4D) and H^+ (Fig. 4E) in the presence of EMPA is present and similar to recovery with DMSO. Accordingly, the summarized slopes of H^+ recovery were not different



between EMPA and DMSO treated cells (Fig. 4F).

Figure 4 EMPA inhibits NHE1 activity in H9c2 cells, with loss of NHE1 inhibition after PXIV treatment. H9c2 cells not treated with PXIV versus H9c2 cells treated for 10 min with PXIV. (A-C) Average curve changes of intracellular pH (A), $[H^+]$ and slope fitting (B) and summary data of slope (C) for H9c2 cells not treated with PXIV (n=6(DMSO)/8(EMPA) cells from 7 independent experiments, Mann-Whitney test). (D-F) Average curve changes of intracellular pH (D), $[H^+]$ and slope fitting(E) and summary data of NHE1 activity (F) for H9c2 cells treated with 10 min PXIV incubation during NH_4^+ pulse and the pH recovery period (n=7/8 cells from 7 independent

experiments, Mann-Whitney test). EMPA: Empagliflozin. P: PXIV. Slope ($\Delta[H^+]/\Delta s$) is the linear fit over first 50 s of intracellular [H^+] recovery. ns $P > 0.05$, *** $P < 0.001$.

Finally, we examined whether PXIV treatment also affects NHE1 inhibition by the classical inhibitor of NHE1, cariporide. H9C2 cells were subjected to 10 µM cariporide with or without PXIV treatment during and after the $NH4^+$ pulse (supplementary Figure 4). Suppl. Fig. 4A-C clearly illustrates that NHE1 inhibition by cariporide is unaffected by PXIV.

Discussion

The key findings of this study are as follows: 1) Protease XIV can completely abrogate the inhibition of NHE1 by empagliflozin in primary cardiomyocytes and cultured cardiomyoblast cells, and 2) Empagliflozin's inhibition of NHE1 is independent of extracellular pH levels ranging from 7.0 to 7.4. These results highlight the significance of experimental conditions in generating reproducible data and emphasize the careful selection of enzymes used in cell isolation procedures tuned to the specific mechanism under investigation.

Enzymes used for cardiac cell isolation procedures for NHE1 research

In 2017 we were the first to report the inhibition of NHE1 activity by empagliflozin in isolated cardiac cells from rabbit hearts[8]. In that study, cardiac cells were isolated by a 30-minute perfusion of the isolated rabbit heart with collagenase B (15 mg/100ml), collagenase P (5 mg/100ml), hyaluronidase (20 mg/100ml) and a trypsin inhibitor (10 mg/ml), followed by 25-minute incubation of the cells with a similar enzyme solution. In a subsequent study, cells were obtained from isolated mouse hearts by perfusing with liberase TM (0.032 mg/mL) and elastase (1.6 U/mL) for a duration of 15 minute[9]. A similar isolation procedure was used to obtain isolated mouse cardiomyocytes to explore the potential impact of insulin on NHE1 inhibition by empagliflozin[19]. Strong inhibition NHE1 activity by SGLT2i was observed in all these studies. Additionally, NHE1 inhibition by empagliflozin in isolated mouse cardiomyocytes was also observed by Trum and co-workers[20] employing liberase and trypsin as digestive enzymes, and by Peng and co-workers[11] employing trypsin and collagenase enzymes. In contrast, isolated rat cardiomyocytes obtained from perfusion of the isolated rat heart with protease VIX (0.025-0.09 mg/ml) and collagenase type II (0.9-1.0 mg/ml) did not show NHE1 inhibition by SGLT2i [17,21]. The present work demonstrated that exposing rabbit cardiomyocytes during the enzymatic dissociation protocol for 55 minutes to a low dosage of PXIV (0.009 mg/ml), abolished the inhibitory effects of EMPA on NHE1. Similarly, a treatment of only 10 minutes with 0.009 mg/ml PXIV was sufficient to eliminate EMPA's effects on NHE1 in H9c2 cells. Therefore, the use of PXIV may likely explain the absence of NHE1 inhibition in some studies involving isolated cardiomyocytes. It has been demonstrated that the protease XXIV selectively degrades the S5-pore extracellular linker of the KCNH2 (hERG1) protein, which is responsible for the rapidly activating delayed rectifier K^+ current[18]. Docking studies have suggested that SGLT2 inhibitors interact with the extracellular Na^+-binding site of NHE. Further research is required to elucidate the precise molecular domain of the NHE protein that is affected by PXIV proteolysis. Specifically, it is important to determine whether the extracellular Na^+-binding site or another extracellular region of NHE1 is affected. Notably, the inhibition of NHE1 activity by cariporide is not affected by PXIV treatment, indicating different molecular motif for NHE1 inhibition by cariporide versus SGLT2i. Even if the exact molecular mechanism(s) of the effect of proteases are unknown, this study emphasizes that

study methods are key to the interpretation of the results. We speculate that our observations potentially are applicable to the function of other extracellular sarcolemma-bound proteins as well.

Persistent NHE1 inhibition by EMPA at different extracellular pH levels

In the first reports demonstrating NHE1 inhibition by SGLT2 inhibitors, the extracellular pH during measurements at 37 °C was set at 7.2[8,9], whereas this pH equaled 7.4 in reports showing absence of NHE1 inhibition by EMPA[17]. A previous study indicated a potential decrease in NHE1 inhibition by EMPA at higher pH levels[22,23] . Because NHE1 activity is critically dependent on extracellular pH, with extracellular acidosis decreasing NHE1 activity[24,25], we also changed pH during our studies (extracellular pH 7.0-7.4). The results clearly demonstrate that NHE1 inhibition was consistently present at all tested pH levels, with no decreased inhibition at higher extracellular pH.

In conclusion, robust inhibition of the NHE1 by EMPA was observed in non-PXIV treated rabbit cardiomyocytes and rat H9c2 cardiomyoblast cells. However, prolonged exposure to PXIV in cardiac cells abolished NHE1 inhibition by EMPA, highlighting the need for caution when using of PXIV in cardiac cell isolation procedures to examine the effects of SGLT2 inhibitor on cardiac NHE1 activity.

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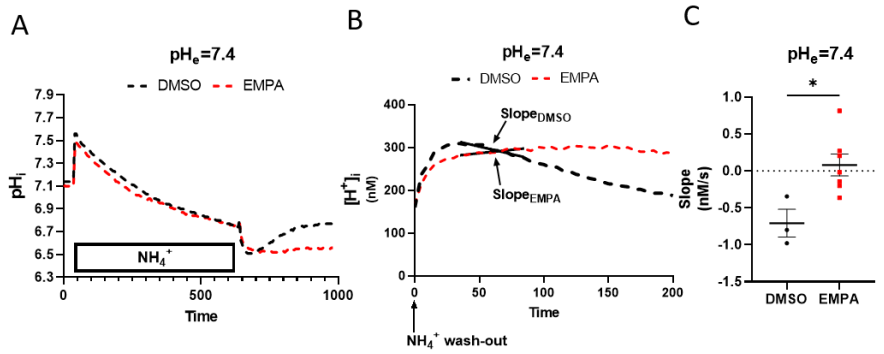
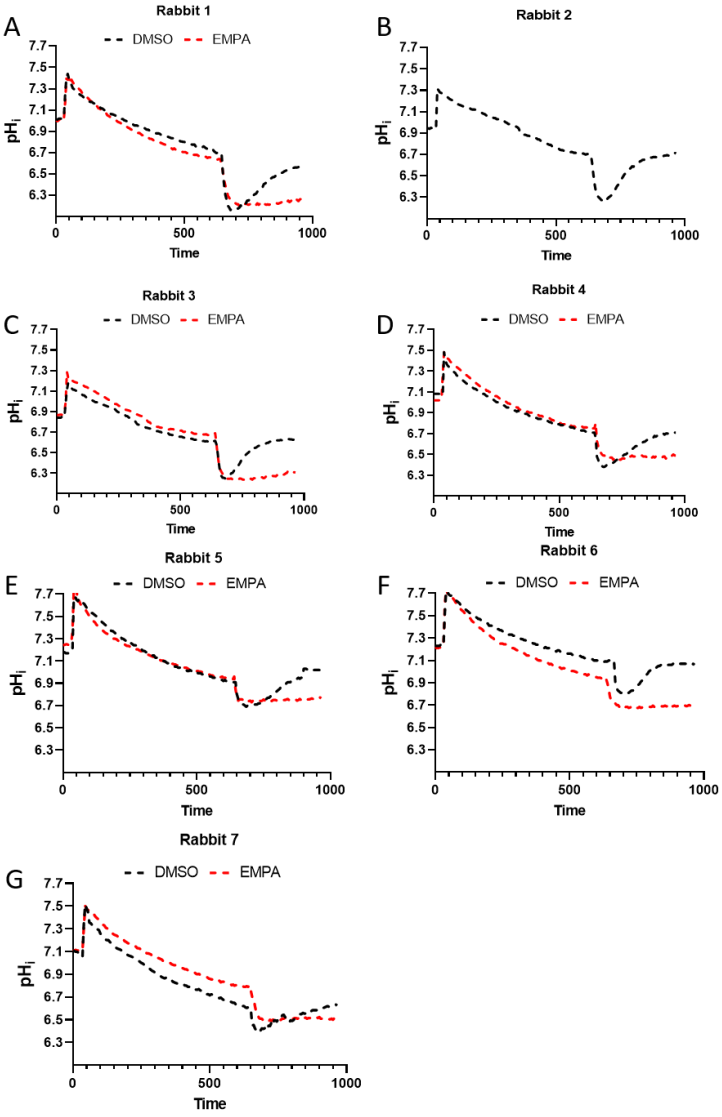
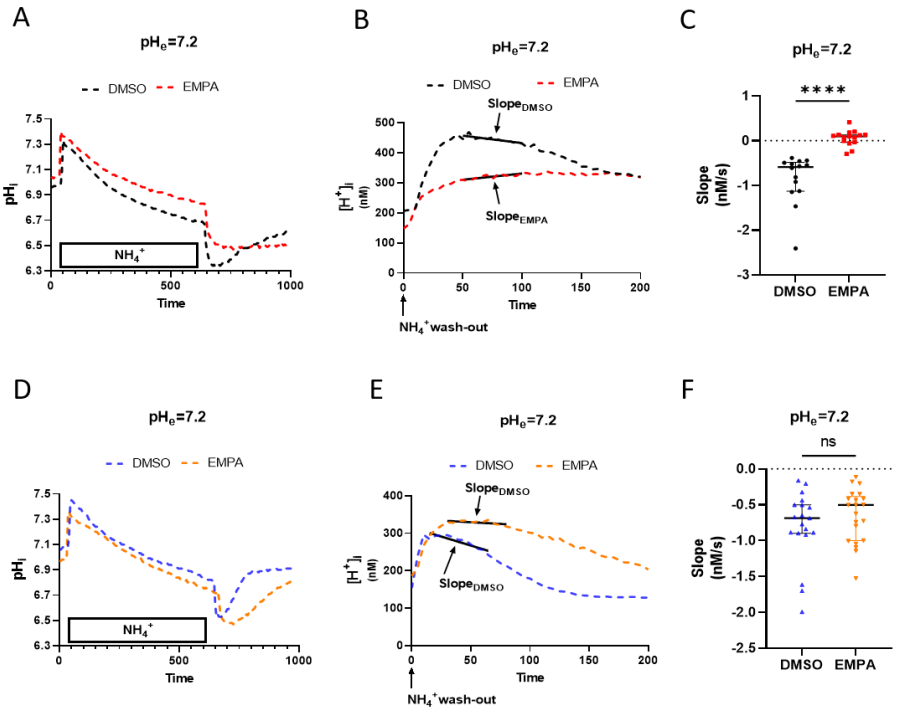
Supplementary



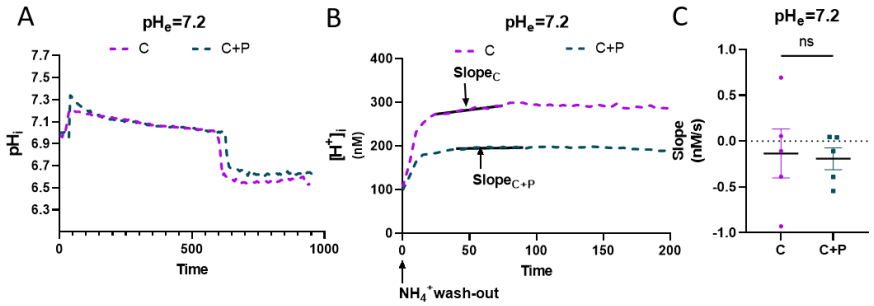
Supplementary figure 1. Empagliflozin inhibits NHE1 activity in rabbit CM isolated in the absence of PXIV. (A) Typical example of intracellular pH tracings for DMSO and EMPA-treated cells isolated without PXIV treatment in response to a NH_4^+ pulse; (B) Typical example of intracellular $[H^+]$ during the first 200 sec after NH_4^+ wash-out in control (DMSO) and EMPA-treated cells, showing the slope of the linear fit of the first 50 sec intracellular $[H^+]$ recovery; (C) NHE1 activity of control (DMSO) and EMPA-treated cells at an extracellular pH of 7.4, as determined by the slope of intracellular $[H^+]$ recovery in rabbit ventricular myocytes isolated without PXIV. (n=3/7 cells from 3/4 rabbits, Paired t test (two tailed)). EMPA: Empagliflozin. Slope ($\Delta[H^+]/\Delta s$) is the linear fit over first 50 s of intracellular $[H^+]$ recovery. * $P < 0.05$.



Supplementary figure 2. Empagliflozin inhibits NHE1 activity in rabbit CMs exposed to PXIV for 35 minutes during the enzymatic protocol. (A-G) Intracellular pH curves in response to a NH_4^+ pulse for DMSO- and EMPA-treated CMs exposed to 35 minutes PXIV during enzymatic dissociation for each of the 7 rabbits, respectively: rabbit1 (2/2 cells); rabbit2 (2/0 cells), no successful EMPA measurements were performed for this rabbit; rabbit3 (3/4 cells); rabbit4 (1/1 cells); rabbit5 (1/2 cells); rabbit6 (1/1 cells); rabbit7 (2/2 cells). EMPA: Empagliflozin.



Supplementary figure 3. Empagliflozin does not inhibit NHE1 activity in rabbit CMs exposed to PXIV for 35 minutes during enzymatic dissociation + 20 min subsequent PXIV incubation. (A-C) Typical example of intracellular pH for DMSO and EMPA treatment after an ammonium pulse (A), $[H^+]$ and slope fitting (B) and summary data (C) for DMSO and EMPA-treated CMs exposed to 35 minutes PXIV at pH 7.2 (n=14(DMSO)/15(EMPA) cells from 5 rabbits, Mann-Whitney test). (D-F) Typical example of intracellular pH (D), $[H^+]$ and slope fitting (E) and summary data (F) for DMSO and EMPA-treated CMs exposed to PXIV for 35 minutes during enzymatic dissociation + 20 min subsequent PXIV incubation at extracellular pH 7.2 (n=19/21 cells from 5 rabbits, Mann-Whitney test). EMPA: Empagliflozin. Slope ($\Delta[H^+]/\Delta s$) is the linear fit over first 50 s of intracellular $[H^+]$ recovery. ns $P > 0.05$, **** $P < 0.0001$.



Supplementary figure 4. NHE1 inhibition by the NHE1 inhibitor cariporide is unaffected by PXIV treatment. (A-C) Average curve changes of intracellular pH (A), $[H^+]$ and slope fitting (B) and summary data (C) of NHE1 activity for H9c2 cells treated for 10 min with 10μM Cariporide with or without PXIV during NH_4^+ pulse and the pH recovery period (n=5/5 cells from 5 independent experiments, Mann-Whitney test). C: Cariporide. C+P: Cariporide+ PXIV. Slope ($\Delta[H^+]/\Delta s$) is the linear fit over first 50 s of intracellular $[H^+]$ recovery. ns $P > 0.05$.