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Role of phosphorylation of Thr^{17} residue of phospholamban in mechanical recovery during hypercapnic acidosis

Cecilia Mundiña-Weilenmann[a], Paola Ferrero[a], Matilde Said[a], Leticia Vittone[a], Evangelia G. Kranias[b], Alicia Mattiazzi[a,*]

[a]*Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, 60 y 120, (1900) La Plata, Argentina*
[b]*Department of Pharmacology and Cell Biophysics, University of Cincinnati, College of Medicine, Cincinnati, OH 4567-0575, USA*

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Abstract

Objectives: To assess the time course of phosphorylation of phospholamban residues, the underlying mechanisms determining these phosphorylations, and their functional impact on the mechanical recovery during acidosis.
Methods: Langendorff perfused rat hearts were submitted to 30 min of hypercapnic acidosis. Contractility, relaxation, and phosphorylation of phospholamban residues, immunodetected by specific antibodies, were determined.
Results: Acidosis produced a mechanical impairment followed by a spontaneous recovery, most of which occurred within the first 3 min of acidosis (early recovery). During this period, contractility and relaxation recovered by 67±9% and 77±11%, respectively, from its maximal depression, together with an increase in the Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII)-dependent phosphorylation of Thr^{17}. The CaMKII inhibitor KN-93, at 1, 5 and 10 μM, decreased Thr^{17} phosphorylation to basal levels and produced a similar impairment of the early relaxation recovery (50%). However, only 5 and 10 μM KN-93 inhibited the early contractile recovery and completely blunted the late mechanical recovery. Inhibition of the reverse mode of the Na^+/Ca^{2+} exchanger by KB-R7943 decreased Thr^{17} phosphorylation but accelerated the early contractile recovery.
Conclusions: CaMKII-dependent Thr^{17} phosphorylation significantly increased at the beginning of acidosis, is responsible for 50% of the early relaxation recovery, and is linked to the activation of the reverse Na^+/Ca^{2+} mode. The early contractile recovery and the late mechanical recovery are dependent on CaMKII but independent of the phosphorylation of the Thr^{17} residue of phospholamban. The reverse Na^+/Ca^{2+} mode has an additional negative effect that opposes the early mechanical recovery.

Keywords: Myocardial acidosis; Phospholamban phosphorylation; CaMKII; Na^+/Ca^{2+} exchanger; Contractile function

1. Introduction

The impairment of contractility and relaxation that occurs when the heart is submitted to acidosis, is followed by a partial mechanical recovery, that occurs in spite of the persistent acidosis [1–6]. The cause of this recovery remains still undefined. Possible mechanisms are a partial recovery of intracellular pH, observed in several experimental models [7,8], and an increase in Ca^{2+} transient amplitude, that would tend to overcome the decrease in Ca^{2+} responsiveness of the contractile proteins produced by a low intracellular pH [9]. The increase in intracellular Ca^{2+} transient has been attributed to two main mechanisms: a displacement of Ca^{2+} from intracellular buffering sites and an increase in intracellular Na^+, due to the acidosis-induced activation of the Na^+/H^+ exchanger (NHE) [3,8,10] and inhibition of the Na^+/K^+-ATPase [11]. This would lead to a decrease in Ca^{2+} efflux or an increase in Ca^{2+} influx, via the Na^+/Ca^{2+} exchanger (NCX) [3,8,10].

Another mechanism that may contribute to the enhancement in Ca^{2+} transient during acidosis, is an increase in the

* Corresponding author. Tel./fax: +54 221 483 4833.
E-mail address: ramattia@atlas.med.unlp.edu.ar (A. Mattiazzi).

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activity of the cardiac sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a), produced by an increase in the phosphorylation of phospholamban (PLN), the major regulator of SERCA2a. In the non-phosphorylated state, PLN tonically inhibits SERCA2a activity and SR Ca^{2+} transport. Phosphorylation of PLN, by either cAMP-dependent protein kinase (PKA), at Ser^{16} residue or Ca^{2+}-calmodulin-dependent protein kinase (CaMKII), at Thr^{17} residue, relieves this inhibition, increasing the affinity of the pump for Ca^{2+} [12]. This would compensate the direct detrimental effect of acidosis on SERCA2a [13–15], leading to an increase in SR Ca^{2+} uptake and an enhancement of SR Ca^{2+} load and release. In support to this hypothesis, a different type of evidence indicates that the SR, particularly PLN and its Thr^{17} site, may be involved in the recovery of contractility that occurred during acidosis: Experiments from our own and other laboratories showed that the mechanical recovery during acidosis, does not take place in the presence of ryanodine and/or thapsigargin, to functionally disable the SR, and can be prevented with the CaMKII inhibitor KN-93 [3,5,6,8]. In addition, the mechanical recovery during acidosis was absent in myocytes from PLN-KO mice [6]. Furthermore, although earlier experiments indicated that phosphorylation of Ser^{16} of PLN was mandatory for the phosphorylation of Thr^{17} site [16–18], experiments from our own laboratory showed that Thr^{17} site of PLN may be phosphorylated independently of Ser^{16} residue, under special conditions of high intracellular Ca^{2+} (which would activate CaMKII) and inhibition of PP1, the phosphatase that dephosphorylates PLN [19–21]. These two requisites are met by acidosis [14,22]. Indeed, an increase in the phosphorylation of Thr^{17} of PLN has been described by us, at the beginning of reperfusion after ischemia, a situation of high intracellular Ca^{2+} associated with a still unrecovered low intracellular pH [23,24]. Under these conditions, the increase in Thr^{17} phosphorylation favored the mechanical recovery [25]. Taken together, these studies support a significant role of PLN, specifically Thr^{17} phosphorylation, on the mechanical recovery from acidosis. However the time course of phosphorylation of PLN residues, the underlying mechanisms of this phosphorylation and its functional impact during the recovery from acidosis, have not been previously studied. The main goal of the present study was to explore this issue.

2. Methods

2.1. Heart perfusions

Animals used in this study were maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). Experiments were performed in isolated hearts from male Wistar rats (200–300 g body wt) perfused according to the Langendorff technique at constant temperature (37 °C), flow (12 ml/min) and heart rate (240 beats/min) as previously described [19]. The composition of the physiological salt solution was (in mM): 128.3 NaCl, 4.7 KCl, 1.35 $CaCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 1.1 $MgCl_2$, 11.1 glucose and 0.04 Na_2EDTA; this solution was equilibrated with 95% O_2–5% CO_2 to give a pH of 7.4 (control solution). The mechanical activity of the heart was assessed by passing into the left ventricle, a latex balloon connected to a pressure transducer. The balloon was filled with aqueous solution to achieve a left ventricular end-diastolic pressure of 7–13 mmHg. Mechanical activity of the left ventricle was evaluated by the developed pressure (LVDP) and the maximal rate of pressure development (+dP/dt). Relaxation was assessed by the time constant of the developed pressure decay (Tau). In some experiments, epicardial monophasic action potentials (mAP) were obtained by using an Ag/$AgCl_2$ suction electrode apposed to the left ventricle [26].

2.2. Experimental protocol

After stabilization at pH 7.4, hearts were perfused with a solution equilibrated with 80% O_2–20% CO_2 (pH 6.8). Hearts were freeze-clamped at different times during acidosis for biochemical assays. When drugs were used, they were perfused 10 min before the beginning of acidosis and throughout the acidosis period (see Results).

2.3. Preparation of SR membrane vesicles

SR membrane vesicles were prepared as previously described [19]. Protein was measured by the method of Bradford using bovine serum albumin as standard. The yield was 1–2 mg membrane vesicles protein/g cardiac tissue.

2.4. Electrophoresis and Western Blot analysis

For immunological detection of phosphorylated PLN, SDS-PAGE was performed using 10% acrylamide slab gels according to Porzio and Pearson, as previously described [19]. 20 μg of membrane proteins were electrophoresed per gel lane. Separated proteins were transferred to PVDF membranes (Immobilon-P, Millipore) and probed with antibodies raised against PLN (1:1000, Affinity BioReagents, USA) or against a PLN peptide (residues 9–19) phosphorylated either at Ser^{16} or at Thr^{17} (1:5000) (Badrilla, West Yorkshire, UK). Immunoreactivity was visualized by peroxidase-conjugated antibodies, using a peroxidase-based chemiluminescence detection kit (ECL, Amersham). The signal intensity of the bands on the film was quantified using Scion Image software (based on NIH Image). Membrane vesicles isolated from hearts perfused with 30 nM isoproterenol, a concentration that produces the maximal inotropic response and phosphorylation of PLN residues [19], were run in parallel with each experimental series. The results are expressed as percentage of the isoproterenol-induced Ser^{16} and Thr^{17} phosphorylation.

2.5. Statistics

Data are expressed as the mean±SEM. Statistical significance was determined by Student's *t*-test for paired or unpaired observations as appropiate, and ANOVA when different groups were compared. The Newman-Keuls test was used to examine statistical differences observed with the ANOVA. A P value<0.05 was considered statistically significant.

3. Results

3.1. Time course of the mechanical recovery during acidosis

The typical inotropic response to respiratory acidosis in perfused rat hearts, is shown in Fig. 1A. Hypercapnic acidosis produced an impairment of contractility and relaxation followed by an intrinsic spontaneous recovery, that occurred in spite of the persistent extracellular acidosis. Fig. 1B to D, shows the overall mechanical results of these experiments. After the rapid decrease in left ventricular developed pressure (LVDP), by 78.9±1.2% from control, (Fig. 1B), and maximal rate of rise of pressure (+dP/dt), by 75.4±0.9% from control, (Fig. 1C), there was a contractile recovery. The increase in LVDP and +dP/dt followed a monoexponential time course. On average, contractility recovered to 57.8±4.4% and 58.6±3.5% of control (LVDP and +dP/dt, respectively). Most of this recovery (approximately 60%) occurred during the first 3 min of acidosis. Acidosis also produced an initial prolongation of the time constant of pressure decline (Tau) by 25.0±2.1 ms from control, after which, Tau decreased monophasically (Fig. 1D). Beyond 5 min, Tau remained virtually stable and significantly higher than control values. Of note, the relaxation recovery was more complete than the contractile recovery and again most of it occurred in the first 3 min of acidosis. The mechanical recovery during acidosis was completely abolished by the presence of ryanodine plus thapsigargin. Under these conditions, acidosis significantly decreased LVDP by 72.0±2.9% from control. This contractility decrease did not recover and after 30 min of acidosis, LVDP was still 77.3±0.4% lower than control. In contrast, the presence of ryanodine plus thapsigargin did not preclude the positive inotropic effect of elevating extracellular calcium from 1.35 to 3.85 mM. High calcium significantly enhanced LVDP by 61.3±6.0% from control, (n=3). Taken together, these results would suggest that the

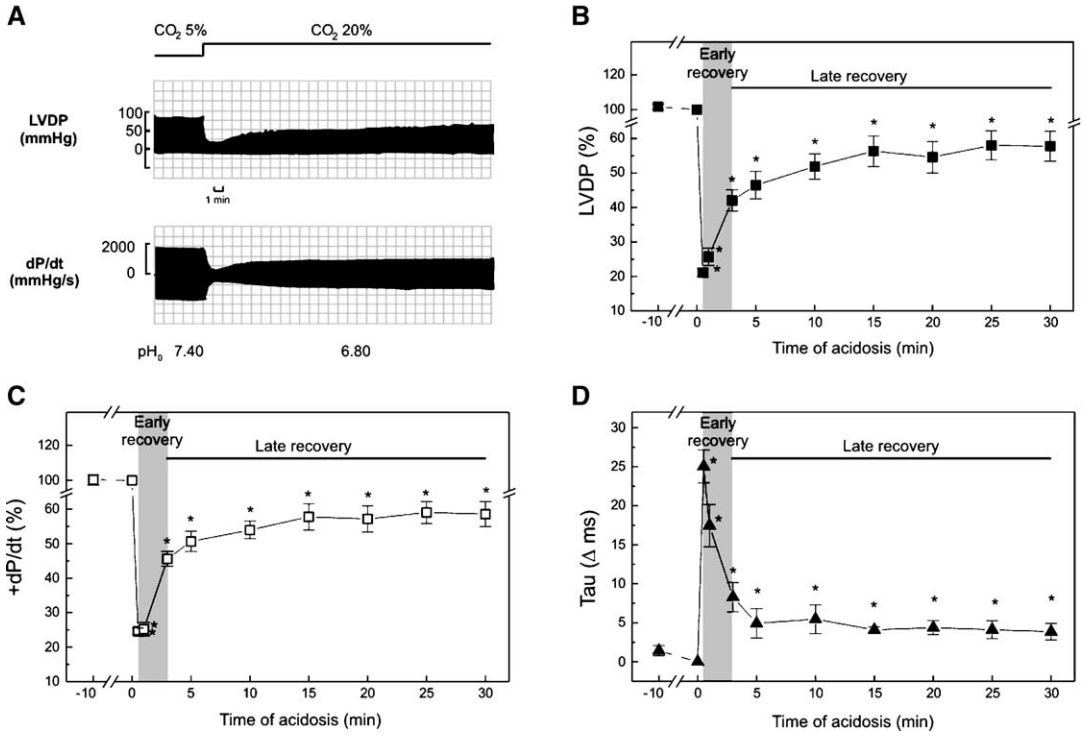

Fig. 1. Effect of acidosis on the left ventricular function. A. Representative continuous records of the effect of hypercapnic acidosis on isovolumic left ventricle developed pressure (LVDP) and its first derivative (dP/dt) in a perfused rat heart. B–D. Average data of LVDP (B), maximal rate of rise of pressure (+dP/dt, C) and the time constant of left ventricular developed pressure decay (Tau, D), of 7 hearts submitted to hypercapnic acidosis. After the initial acidosis-induced negative inotropic and antirelaxant effects (30 s), there is a mechanical recovery that follows a monoexponential time course of the form $y=y_0+A_1(1-e^{-x/t_1})$. The values of the different constants are: y_0=15.6%, A_1=40.8% and t_1=3.3 min for LVDP, y_0=15.9%, A_1=41.9% and t_1=2.8 min for +dP/dt and y_0=33.8 ms, A_1=−29.4 ms and t_1=1.3 min for Tau. LVDP and +dP/dt are expressed as percentage of basal values. Tau is expressed as Δ msec of basal values. * indicates P<0.05 with respect to basal values.

mechanical recovery from acidosis either resides in or at least needs a functionally intact SR, in agreement with previous findings [3,8]. In a group of experiments, epicardial monophasic action potentials were obtained before and at 3 min of acidosis. In agreement with previous results [27], acidosis produced a prolongation of the duration of the action potential at 50% repolarization (APD_{50}) of 9.5±0.6%, with no significant changes in APD_{30} and APD_{90}.

To facilitate the description of the results, the initial period of acidosis, between 30 s and 3 min, during which most of the mechanical recovery occurred, will be referred to as "early recovery". The remainder period of acidosis, during which the mechanical recovery reached steady state, will be referred to as "late recovery".

3.2. Time course of the phosphorylation of PLN residues during acidosis

Fig. 2 shows immunoblots of a typical experiment (Fig. 2A) and the overall results (Fig. 2B) of the time course of the phosphorylation of PLN residues during acidosis. Whereas phosphorylation of Ser^{16} did not significantly change during acidosis, phosphorylation of Thr^{17} started to rise as early as 30 s after the onset of acidosis, reached a maximum at 1 min and remained at high levels until the 3rd min of acidosis. Phosphorylation of Thr^{17} then decreased to levels not significantly different from controls. To better compare the time course of Thr^{17} phosphorylation with that of the contractile and relaxation recoveries, the average results of +dP/dt and Tau during the first 10 min of acidosis are depicted in Fig. 2C. These results demonstrate that the early recovery of contractility and relaxation occurs closely associated to the highest phosphorylation levels of Thr^{17} residue, suggesting that phosphorylation of Thr^{17} may play a role in the mechanical recovery that occurs during acidosis.

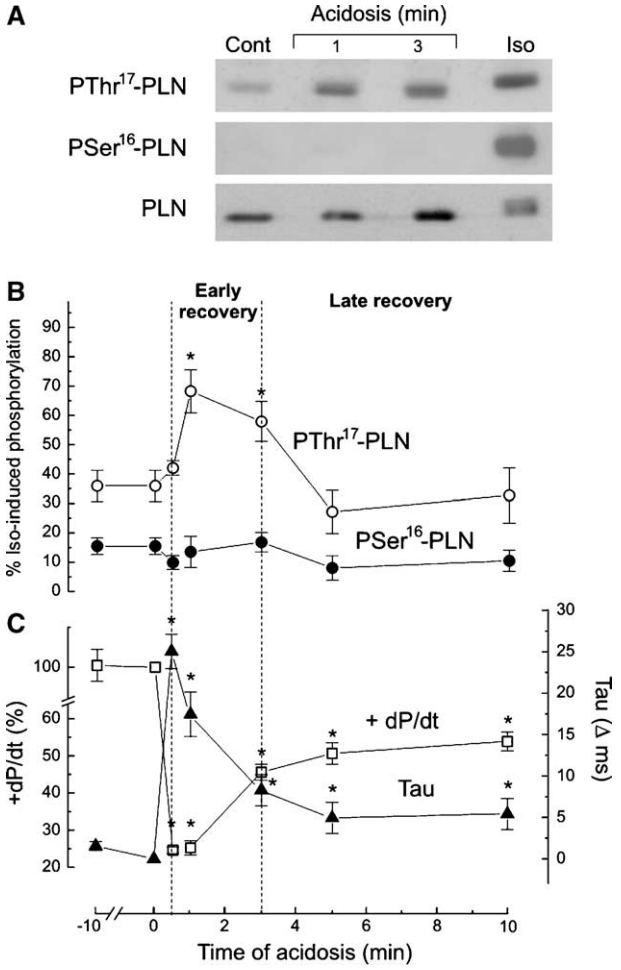

Fig. 2. Effect of acidosis on the phosphorylation of PLN sites. A. Representative immunoblots of SR membrane vesicles isolated from hearts submitted to different times of acidosis, and probed with anti $PSer^{16}$–PLN, anti $PThr^{17}$–PLN and anti PLN. B. Overall results of the densitometric analysis of Ser^{16} and Thr^{17} phosphorylation in immunoblots of SR membrane vesicles isolated from 3–21 hearts. C. Overall results of +dP/dt and Tau during hypercapnic acidosis of Fig. 1, are shown for comparison. Phosphorylation of Thr^{17} site of PLN increased simultaneously with the early recovery of contractility and relaxation and then returned to basal levels. Phosphorylation of Ser^{16} remained at basal levels all along the acidosis. PLN content did not change in each sample. Phosphorylation of PLN sites is expressed as percentage of the site-specific phosphorylation achieved by 30 nM isoproterenol. * indicates $P<0.05$ with respect to basal values.

3.3. Effects of inhibition of CaMKII on the phosphorylation of PLN and the mechanical recovery during acidosis

To further study the possible role of Thr^{17} phosphorylation on the mechanical recovery during acidosis, we performed experiments with the CaMKII inhibitor KN-93. One μM KN-93 reduced to basal values the increase in the phosphorylation of Thr^{17} that occurs after 1 and 3 min of acidosis (Fig. 3A). This decrease was associated with a 50% reduction in the recovery of relaxation (Fig. 3B), without any significant change in the early contractile recovery (Fig. 3C). Higher concentrations of KN-93 (5 and 10 μM), failed to produce a more important impairment of Thr^{17} phosphorylation and of the relaxation recovery than that evoked by 1 μM KN-93. However, these high concentrations of KN-93 produced a significant decrease in the early contractile recovery (Fig. 3C). Taken together, these results indicate that: a) The phosphorylation of Thr^{17} site of PLN is responsible for about half of the early recovery of relaxation; and b) CaMKII-dependent mechanisms, different from the phosphorylation of Thr^{17} of PLN, are responsible for most of the early contractile recovery.

Fig. 4 shows the time course of the effects of the inhibition of CaMKII by 5 μM KN-93, on +dP/dt and Tau, all along the acidosis period. KN-93 significantly depressed the recovery of +dP/dt during the entire period of acidosis (Fig. 4A) and inhibited the small but significant late recovery of relaxation (Fig. 4B). This was in spite of the fact that the phosphorylation of Thr^{17} of PLN was already at basal levels during the late recovery. These results suggest that CaMKII-dependent mechanisms other than Thr^{17} phosphorylation are involved in the late contractile and relaxation recoveries during acidosis.

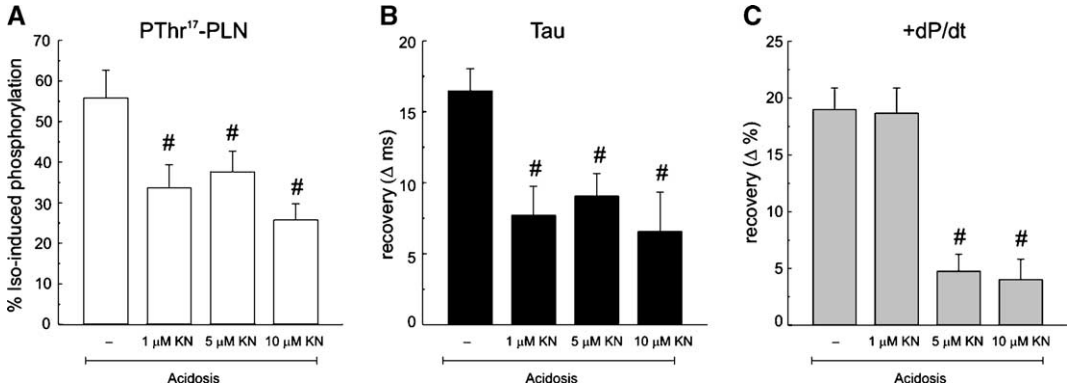

Fig. 3. Effect of CaMKII inhibition on acidosis-induced Thr[17] phosphorylation of PLN and the early contractile and relaxation recoveries. Bars show Thr[17] phosphorylation (A), the recovery of relaxation (Tau, B) and the recovery of contractility (+dP/dt, C) of 14–22 hearts submitted to 3 min of acidosis in the presence and the absence of 1, 5 and 10 μM KN-93 (KN). Whereas 1, 5 and 10 μM KN similarly decreased Thr[17] phosphorylation to basal levels (see Fig. 2) and the recovery of Tau to 50%, only 5 and 10 μM KN significantly depressed contractility. 10 μM KN-93 did not alter basal Thr[17] phosphorylation (32.1±9.0 vs. 20.8±6.6, n=3 respectively). # indicates P<0.05 with respect to acidosis.

3.4. Underlying mechanisms of the phosphorylation of Thr[17] of PLN

Although the Ca^{2+} entry via the NCX seems not to contribute significantly to the activation of contraction under normal conditions [28], an enhancement of this mode of the NCX may occur during acidosis, for different reasons.

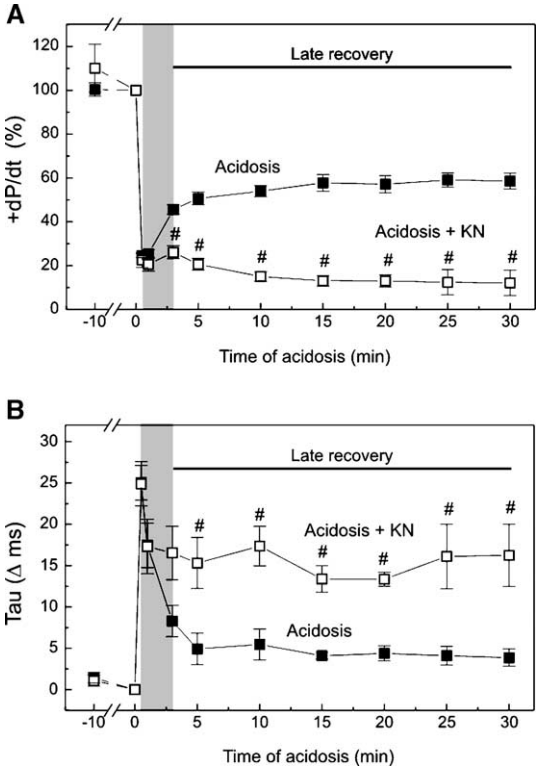

Fig. 4. Effect of CaMKII inhibition on the contractile and relaxation recoveries from acidosis. Overall results of +dP/dt (A) and Tau (B) of 4–7 hearts submitted to acidosis in the presence or the absence of 5 μM KN-93 (KN). KN partially depressed the early recoveries of contractility and relaxation and abolished both late recoveries. # indicates P<0.05 with respect to acidosis.

In the first place, because the acidosis-induced activation of the NHE and inhibition of Na^+–K^+-ATPase would increase intracellular Na^+, which in turn would either slow the forward mode of the NCX or favor the reverse mode. Second, because in our experimental conditions, acidosis produced a prolongation of AP, which would also favor the reverse mode of the NCX. An increase in the influx of Ca^{2+} via the reverse mode of the NCX may be relevant as a mechanism leading to the stimulation of the CaMKII. To test the hypothesis of a possible role of the reverse mode of the NCX on the phosphorylation of Thr[17] of PLN during acidosis, we performed experiments in which acidosis was evoked in the presence of 5 μM KB-R7943 (KB-R), a specific inhibitor of the reverse mode of the NCX. KB-R did not significantly affect Thr[17] phosphorylation prior to acidosis (32.1±9.0 control vs. 17.2±3.6 n=3 KB-R) but significantly decreased to control levels the phosphorylation of Thr[17] residue of PLN observed after 1 and 3 min of acidosis (Fig. 5A). Unexpectedly, KB-R accelerated the early contractile recovery (Fig. 5B) and diminished the initial slowing of the relaxation produced by acidosis: The prolongation of Tau evoked by acidosis was decreased by about half in the presence of the NCX inhibitor, as shown in Fig. 5C. In contrast, blockade of the Ca^{2+} influx mode of the NCX did not significantly affect the late mechanical recovery. Taken together, these results indicate that the activation of the reverse mode of the NCX at the beginning of acidosis, although necessary for the phosphorylation of Thr[17] of PLN, plays an additional detrimental role, which would conspire against the early mechanical recovery.

4. Discussion

An understanding of how pH changes alter cardiac function is important for a better comprehension of some cardiac pathophysiological situations, among which myocardial ischemia is particularly relevant. In man, acidosis

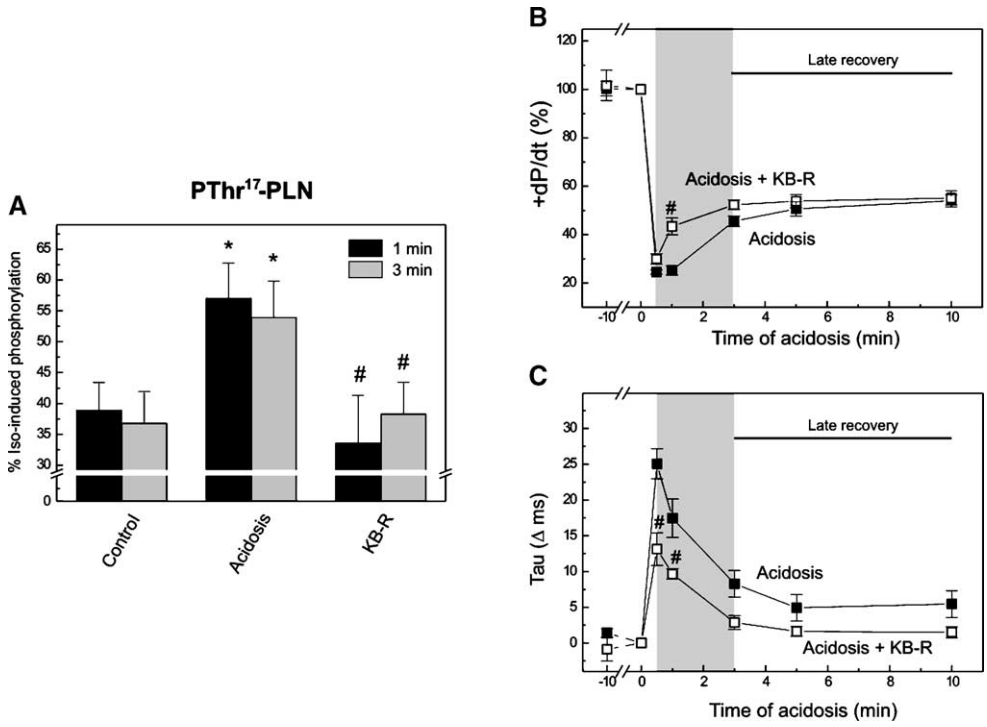

Fig. 5. Effect of KB-R on the acidosis-induced Thr^{17} phosphorylation, and on the contractility and relaxation recoveries. A. Overall results of SR membrane vesicles isolated from hearts (n=6–22) submitted to 1 and 3 min of acidosis in the presence and the absence of 5 μM KB-R 7943 (KB-R). KB-R decreased the acidosis-induced Thr^{17} phosphorylation. B and C. Overall results of +dP/dt (B) and Tau (C) of 3–7 hearts submitted to acidosis in the presence or the absence of KB-R. KB-R limited the antirelaxant effect of acidosis and accelerated the contractile recovery. # indicates $P<0.05$ with respect to acidosis. * indicates $P<0.05$ with respect to control.

can be detected 15 s after the occlusion of the coronary artery and is a major mechanism for the loss of contractility during ischemia [29]. It has been shown in different species, including man, that much of the inhibitory influence of acidosis is caused by a decreased Ca^{2+} sensitivity of myofibrillar proteins [9,30]. However Ca^{2+} handling proteins play also a role in this effect [14]. After the initial fall in contractility there is a spontaneous mechanical recovery, the mechanism of which is poorly understood. The present results were undertaken to better define the cellular processes underlying this spontaneous mechanical recovery of cardiac muscle experiencing acidosis. Different mechanisms proposed to contribute to this recovery, in particular the phosphorylation of Thr^{17} of PLN and the mechanisms involved in this phosphorylation, were investigated. The results provided new insights into the importance of the phosphorylation of PLN residues as well as into the influence of the NCX mechanism on the mechanical recovery during acidosis.

4.1. Role of the phosphorylation of Thr^{17} site of PLN on the mechanical recovery during acidosis

The current experiments described for the first time, the time course of PLN phosphorylation during the mechanical recovery from acidosis. Phosphorylation of Thr^{17} site of PLN transiently increased at the onset of acidosis and accounted for nearly 50% of the relaxation recovery, most of which occurred within the first 3 min of acidosis. This phosphorylation would provide a mechanism to overcome the direct depressant effect of acidosis on SERCA2a [15]. The tight association between PLN phosphorylation and myocardial relaxation [21] might explain the finding that most of the relaxation recovery occurs during the first 3 min of acidosis. In contrast to the important role of Thr^{17} phosphorylation in the early relaxation recovery, the phosphorylation of this residue has no influence on the early contractile recovery, as should be expected. The reasons for this finding are not clear, but might reflect the still unrecovered activity of ryanodine receptors (RyR), from the direct inhibitory effect of intracellular acidosis [31].

Thr^{17} site became dephosphorylated after 5 min of acidosis, which would indicate that, in our experimental conditions, the phosphorylation of this site does not contribute to the late contractile and relaxation recoveries. A more prominent role of the phosphorylation of PLN residues might be expected in vivo. Systemic acidosis is known to increase sympathetic nerve activity [32]. Thus, through the stimulation of the β-adrenergic receptors under these conditions, the phosphorylation of both, Thr^{17} and Ser^{16} residues of PLN is likely to occur [16,17,19,21]. Recent experiments by DeSantiago et al. [6] in PLN knock out mice (PLN-KO), demonstrate that myocytes lacking PLN failed to show any significant mechanical recovery. These results would indicate that the presence of PLN, as

opposite to the chronic de-repression of SERCA2 in PLN-KO mice, is required throughout the entire acidosis period for both, the relaxation and contractile recoveries. The present results further extend these findings demonstrating, by direct assessment of PLN phosphorylation sites, that PLN phosphorylation at Thr^{17} increased at the onset of acidosis and is responsible for about half of the relaxation recovery.

4.2. Mechanisms involved in the phosphorylation status of Thr^{17} of PLN during acidosis

As stated above, one of the main mechanisms proposed to explain the mechanical recovery from acidosis, involves a sequence of events including the increase in intracellular Na^+ which, by either slowing the forward mode of the NCX and/or favoring its reverse mode, leads to an increase in intracellular Ca^{2+} [3,8,10]. More recently, the mechanical recovery has been attributed to the activity of CaMKII-dependent mechanisms [5,6,33]. The present results provide new pieces of evidence to these proposed mechanisms. We showed that CaMKII-dependent phosphorylation of Thr^{17} site of PLN occurred as a consequence of the Ca^{2+} influx mode of the NCX at the beginning of acidosis. To the best of our knowledge, the present results provide the first evidence for an enhancement of the activity of the reverse NCX mode at the beginning of acidosis. Activation of this mode of the NCX during acidosis may be favored by the increase in intracellular Na^+—due to the activation of the Na^+/H^+ exchanger and the inhibition of the Na^+/K^+-ATPase [3,8,10,11]—and the prolongation of the action potential duration observed in the present results, at 3 min of acidosis. As in the case of the reperfusion after ischemia [25], Ca^{2+} influx at the beginning of acidosis, appears to be detrimental rather than beneficial to the mechanical recovery: The inhibition of the reverse mode of the NCX enhanced the contractile recovery and diminished the initial slowing of relaxation produced by acidosis, even in spite of the fact that the increase in the phosphorylation of Thr^{17}, the main responsible for the relaxation recovery, was abolished. The cause of the deleterious effect of the NCX was not explored in the present experiments. One possible clue to explain these results might lie in the impairment of SR Ca^{2+} transport produced by acidosis [13], which would reduce the capacity of the myocytes to handle an enhanced Ca^{2+} influx through the NCX, in the initial period of acidosis. In this scenario, the phosphorylation of $Thr^{17}$of PLN would counterbalance at least in part, this diminished capacity of SR Ca^{2+} uptake produced by acidosis. This conclusion is supported by the impairment of the relaxation recovery observed when phosphorylation of Thr^{17} of PLN was decreased by specifically inhibiting CaMKII. The present results also indicate that the influx of Ca^{2+} through the reverse mode of the NCX, would only take place at the beginning of the acidosis, since inhibition of this mode of the NCX by KB-R, did not significantly affect the late contractile and relaxation recoveries. However a slowing of the forward mode of the NCX could still contribute to the late contractile recovery during acidosis. The forward mode of the NCX contributes a variable amount towards relaxation or intracellular Ca^{2+} decline, depending on species [28]. In the rat and under normal conditions, this contribution is rather small (below 10%), when compared with SERCA2a [34]. A slowing down of the forward mode of the NCX—due to the acidosis-induced increase in intracellular Na^+—might limit Ca^{2+} extrusion from the cell, contributing to maintain or even increase intracellular Ca^{2+}, in the presence of an impaired SR Ca^{2+} uptake. Although not pursued in the present study, the possibility of a contribution of the forward mode of the NCX to the late mechanical recovery during acidosis, deserves further investigation.

One additional finding of the present results deserved further discussion. Although CaMKII appears to remain active towards substrates different from PLN in the late mechanical recovery, Thr^{17} became dephosphorylated during this period (Fig. 2). A possible explanation to these results may lie in the pH-dependence of phosphatase activity. Previous studies have shown that PP1, the major phosphatase that dephosphorylates PLN has a marked pH-dependence, with its activity increasing as pH increases [22]. Thus, it appears likely that a partial recovery of intracellular pH, triggered by NHE activation, would re-activate PP1 and be responsible for the dephosphorylation of Thr^{17} site. Compartmentalization of CaMKII and different targeting of PP1 and other phosphatases, might explain the CaMKII phosphorylation of other Ca^{2+} handling proteins different from PLN during acidosis.

4.3. Mechanisms underlying the mechanical recovery during acidosis, different from PLN phosphorylation

The CaMKII-dependence of the late contractile recovery, would suggest that proteins different from PLN, also substrates of CaMKII, are involved in this recovery. In vitro studies indicated that both, SERCA2a and the RyR may be phosphorylated by the activation of CaMKII [35–37]. However, the physiological effect of these phosphorylations is rather controversial and far from being understood [35,36,38,39]. In contrast, it has been clearly demonstrated that CaMKII-dependent phosphorylation of L-type Ca^{2+} channels, is a necessary mechanism to overcome the direct depressant effect of acidosis on L-type Ca^{2+}current [38]. Although we have not assessed this issue, it is tempting to speculate that phosphorylation of these channels may contribute to the CaMKII-dependent contractile recovery showed in the present and previous papers [5,6,33].

The role played by Thr^{17} phosphorylation on the early relaxation recovery during acidosis and the mechanisms involved in this phosphorylation, are similar to those evoked by the reperfusion after ischemia, a situation in which acidosis also occurred. As in the case of acidosis, Thr^{17} site of PLN results phosphorylated at the beginning of reperfu-

sion, as a consequence of the increase in intracellular Ca^{2+} through the reverse mode of the NCX and the decrease in intracellular pH [25]. Also in both situations, the phosphorylation of Thr^{17} site of PLN appears to provide a mechanism that favors the mechanical recovery.

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