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Review

Ca^{2+}/calmodulin-dependent protein kinase: A key component in the contractile recovery from acidosis

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Abstract

Intracellular acidosis exerts substantial effects on the contractile performance of the heart. Soon after the onset of acidosis, contractility diminishes, largely due to a decrease in myofilament Ca^{2+} responsiveness. This decrease in contractility is followed by a progressive recovery that occurs despite the persistent acidosis. This recovery is the result of different mechanisms that converge to increase diastolic Ca^{2+} levels and Ca^{2+} transient amplitude. Recent experimental evidence indicates that activation of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is an essential step in the sequence of events that increases the Ca^{2+} transient amplitude and produces contractile recovery. CaMKII may act as an amplifier, providing compensatory pathways to offset the inhibitory effects of acidosis on many of the Ca^{2+} handling proteins. CaMKII-induced phosphorylation of the SERCA2a regulatory protein phospholamban (PLN) has the potential to promote an increase in sarcoplasmic reticulum (SR) Ca^{2+} uptake and SR Ca^{2+} load, and is a likely candidate to mediate the mechanical recovery from acidosis. In addition, CaMKII-dependent phosphorylation of proteins other than PLN may also contribute to this recovery.

Keywords: Acidosis; CaMKII; Protein phosphorylation; SR function

1. Introduction

An understanding of how changes in intracellular pH (pH_i) alter myocardial function is important for a better comprehension of cardiac pathophysiological situations, among which myocardial ischemia is particularly relevant. In humans, acidosis can be detected 15 s after the occlusion of the coronary artery and is a major mechanism for the loss of contractility during ischemia [1] and one of the causes of arrhythmia, a typical clinical characteristic in this disease (see [2] for review). The marked acidosis observed during myocardial ischemia (more than 0.5 pH units) [3] is the result of the accumulation of lactic acid (as a consequence of anaerobic glycolysis) and CO_2 (which can increase fourfold after 30 min ischemia [4]), within the ischemic region. In addition and not less relevant, cellular H^+ metabolism is tightly linked to two other ions that control myocardial contractility, Na^+ and Ca^{2+}, by Na^+–H^+ (NHE) and Na^+–Ca^{2+} (NCX) exchanges and possibly by sharing intracellular buffer sites. This implies a reciprocal influence of pH_i on myocardial contractility and of inotropic agents on pH_i. Indeed, increases in both intracellular Ca^{2+} (Ca_i^{2+}) and in heart rate, have been shown to decrease pH_i [5,6]. Conversely, a fall in pH_i can elevate Ca_i^{2+} as will be described in detail below.

2. Influence of acidosis on myocardial contractility and relaxation

It has been known for over a century that acidosis is associated with a decrease in the ability of the heart to generate tension [7]. This negative inotropic effect is produced mainly by intracellular, rather than extracellular, acidosis [8,9]. Intracellular acidosis causes a rapid decline in the contraction of cardiac muscle, which is primarily attributed to a decrease in myofilament Ca^{2+} responsiveness

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[10,11], although a decrease in the intracellular Ca^{2+} transient due to inhibition of Ca^{2+} release from the sarcoplasmic reticulum (SR) during this period may also occur [12]. The decrease in contractility is associated with a concomitant impairment of relaxation, that takes place in spite of the decrease in the responsiveness of the contractile proteins to Ca^{2+} (which would tend to produce an opposite effect on relaxation) and appears to be mainly evoked by a direct inhibition of SR-Ca^{2+}-ATPase (SERCA2a) [10,13]. The initial impairment of contractility and relaxation is followed by a spontaneous mechanical recovery which occurs despite the persistent acidosis [12,14–24]. Our understanding of the mechanisms of this recovery remains incomplete and several different hypotheses have been proposed. In recent years various laboratories have provided evidence to support a significant role of the activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in this recovery [22–26]. In the present review we consider the possible mechanisms involved in the mechanical recovery from acidosis focusing on the putative role of CaMKII.

3. CaMKII. Structure and regulation

CaMKII is a multimeric holoenzyme composed of 6–12 subunits encoded by 4 separate genes, α, β, γ and δ [27]. This Ser/Thr kinase has diverse roles in virtually all cell types and is regulated by a plethora of mechanisms. The δ isoform predominates in the heart [28] and a splice variant, δc, is localized to the cytosol and phosphorylates several important Ca^{2+} regulatory proteins as described below. Local changes in Ca^{2+} concentration induce activation of CaMKII by binding to calmodulin (CaM). Binding of the Ca^{2+}–CaM complex to an autoregulatory region of the enzyme is presumed to disrupt autoinhibitory interactions, allowing substrates and ATP to gain access to the catalytic domain. Simultaneous Ca^{2+}–CaM binding to adjacent subunits within the same holoenzyme, results in the rapid autophosphorylation of Thr-287 site (Thr-286 for the α isoform of CaMKII). This autophosphorylation has two important regulatory consequences: (1) it produces an approximately 1000-fold increase in the affinity of CaMKII for Ca^{2+}–CaM and (2) it results in autonomous activity, independent of Ca_i^{2+} [27]. Therefore, transient elevations of Ca_i^{2+} can generate a prolonged response through the constitutive activity of autophosphorylated CaMKII, thus, conferring the unique property of "memory" on this enzyme. The activity of CaMKII is also greatly dependent on various other factors. In particular the activity of phosphatases which dephosphorylate CaMKII is of primary importance. Four species of protein phosphatases, PP1, PP2A, PP2C and a novel family of specific CaMKII phosphatases have been reported to dephosphorylate and negatively regulate CaMKII [29]. The activity of CaMKII is further modulated by targeting to specific cellular compartments. Targeting confers on the kinase not only substrate specificity, but also allows CaMKII to be in close proximity to the Ca^{2+} signal to which the

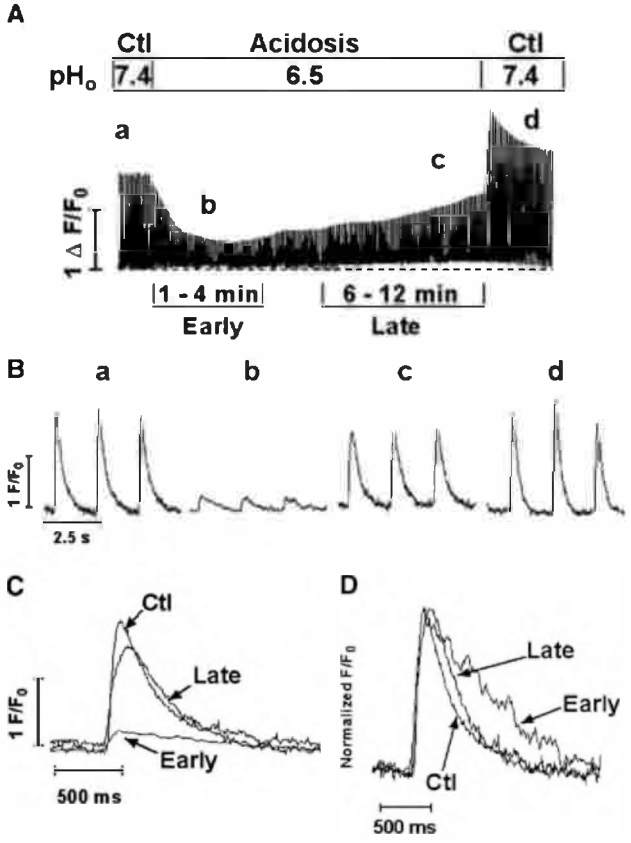

Fig. 1. Influence of acidosis on Ca^{2+} transients in mouse myocytes. A. Isolated myocytes were submitted to acidosis by changing the pH of the HEPES buffer from 7.4 to 6.5 for 12 min. Ca^{2+} transient amplitude was measured using fluo-3 ($\Delta F/F_o$). The initial decrease in the Ca^{2+} transient amplitude was followed by a recovery phase towards control values. Returning to control pH 7.4 produced an overshoot in Ca^{2+} transients that slowly return to control. Dashed line indicates mean level of diastolic Ca^{2+} before acidosis. B. Representative Ca^{2+} transients recorded at higher speed at times corresponding to a–d in A: Control pH 7.4 (a); early acidosis (1–4 min), pH 6.5 (b); late acidosis (6–12 min), pH 6.5 (c); returning to control pH 7.4 (d). C. Superimposed Ca^{2+} transient traces. D. Normalized Ca^{2+} transients to facilitate the comparison of the time course (from DeSantiago et al. [23] with permission).

kinase is intended to respond and determines the type or level of phosphatases to which it is exposed [30]. In cardiomyocytes, CaMKII has been found to be enriched in the T tubules, and to colocalize with ryanodine receptors (RyR2) and L-type Ca^{2+} channels, two of the kinase substrates [31,32]. In the longitudinal SR, CaMKII may regulate SERCA2a either through direct phosphorylation [33,34] and/or through phosphorylation of its regulatory protein phospholamban (PLN) [35]. Interestingly, it has been shown in neurons that the localization of CaMKII in different subcellular compartments may be dynamically regulated, and translocation of the enzyme has been described in these cells [36]. Finally, CaMKII undergoes holoenzyme association ("self-association"). This clustering of cytoplasmic CaMKII into compact aggregates has also been described in neurons under conditions of sustained Ca_i^{2+} elevation and it is favored by decreased levels of ATP and by acidic pH [37,38]. Self-association between holoenzymes would

severely restrict CaMKII targeting and its access to substrates, offering a cellular defense mechanism that would limit CaMKII activation during Ca^{2+} overload [37,38].

4. Possible mechanisms involved in the recovery of contractility during acidosis

There are two main mechanisms potentially involved in the mechanical recovery from acidosis: (1) the increase in Ca_i^{2+} and (2) the recovery of myofilament Ca^{2+} responsiveness. These are not mutually exclusive mechanisms, and there is evidence of CaMKII participation in both.

4.1. The increase in Ca_i^{2+}

Acidosis interferes with many of the systems involved in regulating cardiomyocyte Ca^{2+} handling. Reduced pH_i inhibits the L-type Ca^{2+} current (I_{Ca}), the release of Ca^{2+} by the RyR2 and the activity of SERCA2a [10,12,13,16,39–41]. All these effects might be expected to decrease Ca^{2+} transients. However, although initially the acid load may produce an increased [22,42], decreased [23,41] or unchanged [40] amplitude of the Ca^{2+} transient, later during acidosis a progressive increase in Ca^{2+} transient amplitude as well as in diastolic Ca^{2+} is consistently observed (Fig. 1) [22,23,40–42]. Fig. 1 also shows that the rate of Ca^{2+} transient decline is very slow at the beginning of acidosis, returning towards control values coincident with the recovery of the Ca^{2+} transient amplitude. All these effects have been attributed to different mechanisms triggered by acidosis, which culminate in an increase in diastolic Ca^{2+} and in SR Ca^{2+} content with the consequent enhancement of SR Ca^{2+} release during the twitch. These mechanisms may be either dependent on the acidosis-induced activation of acid-extrusion transporters, which tend to recover pH_i, or independent of the activation of these transporters.

4.1.1. Mechanisms dependent on the activation of acid-extrusion transporters

Steady state pH_i in adult mammalian myocardium is typically 7.1. Intracellular proton concentration is strongly buffered by CO_2-dependent and CO_2-independent intrinsic buffers under normal conditions. The strict regulation of pH_i is further accomplished by well-characterized sarcolemmal ion-transport systems. These transporters include two acid-extrusion mechanisms [the Na^+–H^+ exchanger (NHE) (the only active mechanism in the absence of HCO_3^-) and the Na^+–HCO_3^- symport (NBC)], and two alkali-extrusion mechanisms [the Cl^-–HCO_3^- anion exchanger (AE) and the Cl^-–OH^- exchanger] [43]. Following an acid load, activation of the acid-extrusion mechanisms would tend to recover pH_i during maintained acidosis [19,44,45]. Results by Vandenberg et al. [45] suggested that the recovery of pH_i after an increase in pCO_2 involves both the NHE and the NBC mechanisms. However other pieces of experimental evidence indicated that either this pH_i recovery or the consequent increase in intracellular Na^+ (Na_i^+) was completely blocked by the specific inhibition of the NHE [18–20], suggesting a predominant role of NHE in the recovery from acidosis. In agreement with these results, Leem et al. [43] reported that the NHE is more strongly activated than the NBC in the presence of an acid load. Activation of any of these acid-extrusion pathways will produce an increase in Na_i^+ which would in turn increase Ca_i^{2+} by slowing the forward and/or favoring the reverse NCX mode, with a consequent increase in contractility [18,20,21,46]. Supporting this hypothesis, Kim and Smith [47] reported that blocking NHE with ethylisopropylamiloride (EIPA) abolished the rise in Ca_i^{2+} induced by the washout of NH_4Cl. Moreover, Harrison et al. [20] found a strong correlation between the increase in contractility and Na_i^+ evoked by acidosis, both of which were suppressed by the NHE inhibitor amiloride. Similar findings in relation to the mechanical recovery have been reported by Pérez et al. [21], using low extracellular Na^+ or the amiloride derivative EIPA to inhibit the NHE.

4.1.2. Mechanisms independent of the activation of NHE or NBC

It has been reported that acidosis can increase Ca_i^{2+} even in the absence of extracellular Na^+ or after blocking the NHE, thus challenging the involvement of NHE and NCX in the recovery of Ca^{2+} transient amplitude [41,48]. However, these findings do not exclude the possibility that under physiological conditions both acid-extrusion dependent and independent mechanisms, acting in concert, contribute to the increase in Ca_i^{2+} after an acid load. Among the acid-extrusion independent mechanisms, intracellular Ca^{2+} buffer sites are a possible source for the increase of diastolic Ca^{2+} during acidosis [12]. Displacement of Ca^{2+} from intracellular cation binding sites by H^+ would lead to a rise of diastolic Ca^{2+} and in this way contribute to the increase in SR Ca^{2+} content observed during acidosis [42]. In addition, a direct action of acidosis on SR function would also favor an increase in diastolic Ca^{2+}: as noted above, acidosis decreases the activity of SERCA2a and reduces SR Ca^{2+} uptake [10,13]. This decrease in SR Ca^{2+} uptake may contribute to increasing diastolic Ca^{2+} which in turn would tend to compensate for the decreased activity of SERCA2a [20,40]. Furthermore, the inhibition of SR Ca^{2+} release by acidosis would directly cause a rise in SR Ca^{2+} content [41]. Finally, two additional actions of acidosis, that may contribute to the increase in SR Ca^{2+} load are the inactivation of Na^+–K^+ ATPase [49], which would help to maintain a high level of Na_i^+ and thereby increase diastolic Ca^{2+} by slowing the forward NCX mode (Ca^{2+}-efflux mode), and the inhibition of NCX [50], which would directly contribute to the increase in diastolic Ca^{2+} and therefore SR Ca^{2+} load.

On the basis of the available experimental evidence, the relative importance of these acid-extrusion dependent and independent mechanisms in producing the increase in Ca_i^{2+} during acidosis is difficult to define. Although there are some data that strongly suggest a predominance of the NHE/NCX route [19,20,47], the experimental evidence also indicates that participation of other mechanisms outlined above cannot be dismissed.

4.1.3. The critical role of the SR

While the increase in diastolic Ca^{2+} evoked by the mechanisms discussed above seems to be a necessary step for the recovery of contractility during acidosis, the mechanical recovery does not occur in the absence of a functional SR. It has been demonstrated that the inhibition of the SR function by ryanodine suppresses the mechanical recovery that occurs during acidosis, without significantly affecting the recovery of pH_i and the increase in Na_i^+ and diastolic Ca^{2+} evoked by acidosis [20–22]. Taken together, these results point to the SR as the final effector in the cascade of events that leads to the recovery of Ca^{2+} and contractility during acidosis.

4.2. The recovery of myofilament Ca^{2+} responsiveness

It is generally considered that the contractile recovery during acidosis is mainly due to an increase in Ca_i^{2+}, as discussed above. However, an increase in myofilament Ca^{2+} responsiveness might also take place with the recovery of pH_i and contribute to the contractile recovery. Orchard [12] suggested that the slow recovery of tension that occurs towards the end of the acidosis period with no significant change in the amplitude of Ca^{2+} transients, might represent increased Ca^{2+} binding to myofilaments associated with pH_i recovery. Under some experimental conditions, this pH_i recovery is significant and appears to account at least for a fraction of the contractile recovery [19,45]. Whether this pH_i effect is due to improved myofibrillar Ca^{2+} responsiveness and/or to an increase in Na_i^+ and Ca_i^{2+} following activation of acid-extrusion mechanisms is not clear. However in most of the studies, either a small increase in pH_i, which could not account for the mechanical recovery, or a lack of pH_i recovery was observed [20–22,51–53]. These results would strongly suggest that the relative importance of the recovery of myofilament Ca^{2+} responsiveness due to alterations in pH_i on the mechanical recovery, is modest.

In summary, the main mechanism responsible for the contractile recovery appears to be an increase in Ca_i^{2+}. This increase that occurs in spite of the negative effect of acidosis on the different steps of the excitation–contraction coupling (ECC) process, was attributed to the mechanisms triggered by acidosis discussed above, that would produce an increase in diastolic Ca^{2+} favoring the increase in SR Ca^{2+} load and Ca^{2+} transients. Recently, new evidence has emerged which strongly suggests that activation of CaMKII plays a novel and central role in the mechanical recovery from acidosis. Importantly, this CaMKII involvement appears to be essential to the mechanical recovery and is not simply an additional contributing factor.

5. Effects of an acid load on CaMKII activity

Prior to the discussion of the effect of CaMKII on the recovery from acidosis, we will review the possible effects of acidosis on CaMKII activity. As outlined above, the activity level of CaMKII depends on the balance between the binding of Ca^{2+}–CaM which activates the kinase and induces kinase autophosphorylation and on the activity of the phosphatases which dephosphorylate CaMKII. Consequently, acidosis may alter CaMKII activity in several different ways. First, acidosis could directly modify the binding of Ca^{2+}–CaM and/or the rate of autophosphorylation of the kinase. To the best of our knowledge, there is no detailed information available relating to this issue. Second, acidosis may activate CaMKII by the simultaneous increase in Ca_i^{2+} and inhibition of phosphatases that dephosphorylate it. Indeed PP1, a major phosphatase in the regulation of differentially targeted CaMKIIs involved in the modulation of cardiac ECC [54] has been shown to be inhibited at low pH_i [55]. Third, and as mentioned above, acidosis favors the formation of holoenzyme clusters under conditions of sustained Ca^{2+} elevation, with a negative impact on the activity of CaMKII [37,38]. Thus, the different means by which acidosis may alter CaMKII activity converge into a puzzling scenario, and the net effect of acidosis on the activity of the kinase is difficult to predict. A key finding in resolving this complexity has been the observation that acidosis produces a prominent increase in the CaMKII-dependent phosphorylation of Thr^{17} of PLN [24,25]. In addition, several studies have identified a role for CaMKII in the mechanical recovery from acidosis, based on experimental observations that inhibition of the kinase suppresses mechanical recovery [22–24,26]. These latter results imply that acidosis increases the activity of CaMKII.

6. CaMKII and the regulation of the acid-extrusion mechanisms

The regulation of transporters involved in pH_i control has been extensively investigated in recent years, in particular that of the NHE. In the heart, the NHE-1 is the predominant isoform [56], and in the context of this review the regulation of this exchanger by Ca^{2+} is of special interest. Experimental evidence indicates that Ca^{2+} may control NHE-1 activity by at least two different mechanisms. The first mechanism is via the direct binding of Ca^{2+}–CaM. Both high and low affinity CaM binding sites have been described. The high affinity site may act in an autoinhibitory manner and elevation of Ca_i^{2+}, by permitting CaM binding, may relieve the inhibition and stimulate the activity of the protein [57]. The second mechanism is through the phosphorylation of NHE-1 by CaMKII. *In vitro* experiments by Fliegel et al. [58]

demonstrated that purified CaMKII readily phosphorylates the intracellular cytoplasmic domain of NHE-1, suggesting that the activity of the exchanger may be directly regulated *in vivo* by CaMKII. Consistent with this interpretation, Le Prigent et al. [59] showed that the inhibition of CaMKII under acid load conditions results in a significant reduction of NHE activity in cardiac ventricular myocytes. These results were the first to indicate that the activation of CaMKII may influence the activity of the exchanger and pH_i after an acid load in intact cardiac preparations. However, Komukai et al. [26] failed to detect any significant effect of CaMKII inhibition on the activity of the NHE in cardiac myocytes. Evidently further studies on this issue are warranted. Le Prigent et al. [60] also showed that the activity of NBC was not affected in adult rat myocytes either by decreasing Ca_i^{2+} or by inhibiting CaMKII. Taken together the available evidence indicates that the increase in Ca_i^{2+} during acidosis may activate the NHE directly (relieving the pre-existing inhibition), and possibly also through the activation of CaMKII. The basal phosphorylation and activity of NHE-1 are also regulated by PP1 activity, which has been shown to be constitutively associated with the exchanger [61]. Thus, acidosis may further activate the exchanger by increasing its phosphorylation state through the inhibition of PP1 [55].

7. Influence of CaMKII on the mechanical recovery during acidosis

CaMKII has been implicated in several aspects of acute cellular Ca^{2+} regulation in cardiac cells. CaMKII phosphorylates the L-type Ca^{2+} channel, RyR2, SERCA2a and PLN, and has been associated with the control of various major ECC steps, like Ca^{2+} influx and SR Ca^{2+} uptake and release [26,62]. Moreover, different pieces of evidence, mainly based on the inhibition of CaMKII, strongly suggest a primary role for CaMKII activation in the mechanical recovery after an acid load. It has been shown that acidosis decreases L-type Ca^{2+} current (I_{Ca}) during conventional whole cell patch clamp [63], but has no effect when recording under perforated patch conditions [40]. More recent experiments by Komukai et al. [26] using the latter technique provided some clue to this apparent discrepancy. These authors demonstrated that the inhibitory effect of acidosis on I_{Ca} was unmasked by the presence of 1 μM of KN-93, a specific CaMKII inhibitor and suggested that during conventional whole cell clamp, endogenous CaMKII activity may be inhibited by the Ca^{2+} buffers used with this technique. In contrast, in perforated patch clamp, the acidosis-induced increase in CaMKII would offset the direct inhibitory effect of low pH_i on I_{Ca}, through the phosphorylation of L-type Ca^{2+} channels. Komukai et al. [26] also showed that in the presence of CaMKII inhibition (KN-93), acidosis failed to increase the amplitude of Ca^{2+} transients and caffeine-induced contractures and that the prolongation of the Ca^{2+} transient decay was accentuated. Similar results obtained in different laboratories further confirmed the essential role of CaMKII in the Ca^{2+} transient and mechanical recoveries during acidosis, i.e. in the presence of CaMKII inhibition no recovery occurs [22–24]. A possible explanation for these observations is that acidosis produces an increase in Ca_i^{2+} which, in the presence of the acidosis-induced inhibition of PP1, is sufficient to activate CaMKII. The increased activity of CaMKII would amplify the initial increase in Ca_i^{2+} by promoting the reactivation of SR Ca^{2+} uptake, which in turn would enhance SR Ca^{2+} content and Ca^{2+} transient amplitude [22,23,26].

7.1. Role of CaMKII-dependent PLN phosphorylation

CaMKII may enhance SR Ca^{2+} uptake by two major mechanisms: the phosphorylation of SERCA2a and/or the phosphorylation of PLN. Although a direct CaMKII-dependent phosphorylation and activation of SERCA2a have been described by Narayanan's group [33,34], results from several other laboratories strongly argue against this possibility [64–67]. PLN is an SR protein, which in dephosphorylated state tonically inhibits SERCA2a-mediated SR Ca^{2+} uptake. Phosphorylation of PLN, at either the Ser^{16} site by PKA or at the Thr^{17} site by CaMKII, relieves this inhibition, thus increasing SERCA2a activity and the rate of Ca^{2+} uptake by the SR. Phosphorylation of PLN at Thr^{17} site is therefore a good candidate to explain the effect of CaMKII on the mechanical recovery from acidosis. This possibility is supported by earlier experiments showing that acidosis produced an increase in the phosphorylation of Thr^{17} [25,68] which could be antagonized with BAPTA [25] or inhibited with the CaMKII inhibitor KN-62 [68]. These results support the view that the acidosis-induced increase in Ca_i^{2+} activates CaMKII and phosphorylates Thr^{17} of PLN. This phosphorylation would be further enhanced due to the acidosis-induced inhibition of PP1, the main phosphatase that dephosphorylates PLN [55]. Further support for a major role of PLN in the mechanical recovery during acidosis was provided by the findings by DeSantiago et al. [23], showing that the mechanical recovery during acidosis did not occur in hearts of PLN-knockout (PLN-KO) mice. Although the enhanced SERCA2a activity in PLN-KO mice would constitute an optimal condition for the SR Ca^{2+} uptake during acidosis, the absence of PLN deprives the SR of the necessary mechanism to compensate for the direct inhibitory effect of acidosis on SERCA2a. The results of DeSantiago et al. [23] are consistent with previous findings by Nomura et al. [22]. These authors showed that the mechanical recovery from acidosis did not occur in highly phosphorylated myocytes treated with isoproterenol in combination with a phosphatase inhibitor. In this condition the high phosphorylation level produced by the strong β-adrenergic stimulation and the simultaneous phosphatase inhibition would constitute a situation in which in which no further PLN phosphorylation could be achieved. More recently Mundiña-Weilenmann et al. [24] investigated the time course of the mechanical recovery during acidosis and the associated changes in the phosphorylation of PLN residues. It was

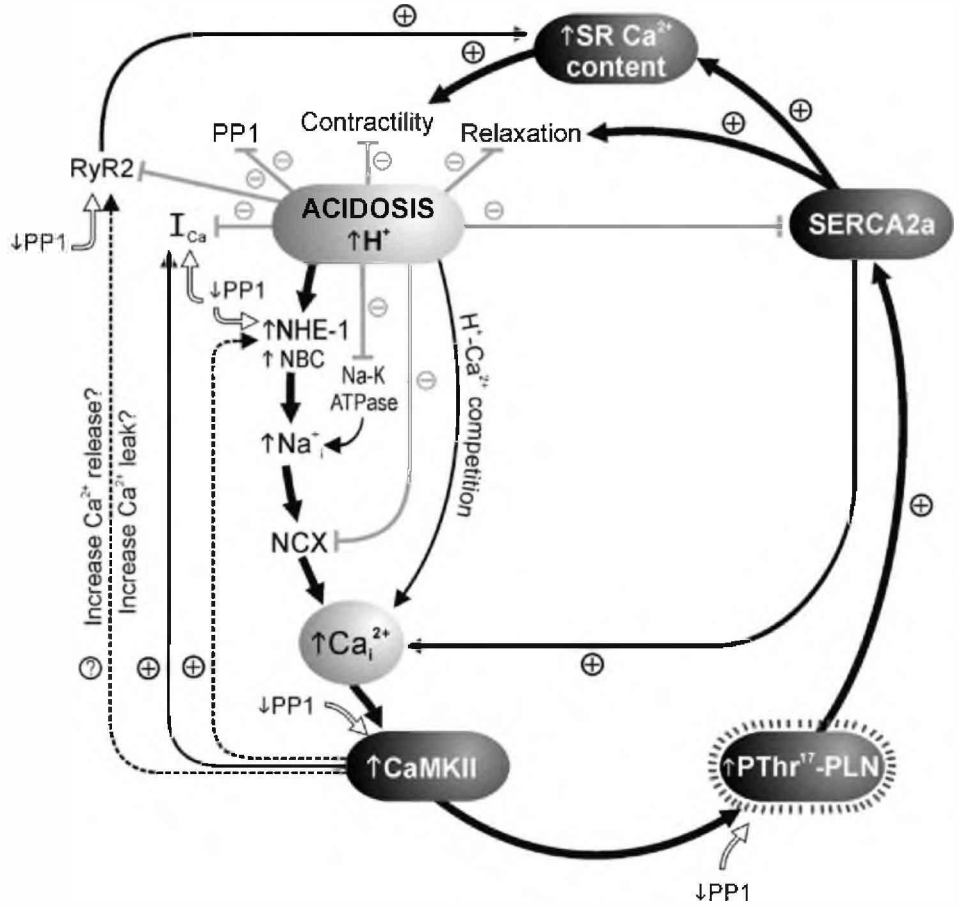

Fig. 2. Schematic representation of potential mechanisms by which CaMKII signaling contributes to the mechanical recovery from acidosis. The increase in Ca_i^{2+} caused by acidosis might be the result of the simultaneous: 1) activation of the acid-extrusion transporters (NHE and possibly NBC) and inactivation of the Na^+–K^+ ATPase which in turn slow the forward mode and/or favor the reverse mode of the NCX; 2) displacement of Ca^{2+} from intracellular buffers; 3) inhibition of NCX; 4) inhibition of SERCA2a. The increase in Ca_i^{2+} will activate CaMKII which, through the phosphorylation of Thr^{17} of PLN ($PThr^{17}$-PLN), would compensate the acidosis-induced inhibitory effect on SERCA2a, leading to the increase in SR Ca^{2+} uptake and SR Ca^{2+} content, with the consequent improvement in myocardial relaxation and contractility. The direct inhibitory effect of acidosis on RyR2 might transiently contribute to the increase in SR Ca^{2+} content. CaMKII-dependent phosphorylation of proteins other than from PLN, might also participate in the increase in Ca_i^{2+} and SR Ca^{2+} content: 1) phosphorylation of L-type Ca^{2+} channels opposes the inhibitory effect of acidosis on I_{Ca}, favoring Ca^{2+} influx to the cell; 2) phosphorylation of NHE could activate the exchanger, contributing to the increase in Ca_i^{2+}. CaMKII phosphorylation of RyR2 has been reported to have controversial effects, which may result in either an increase in SR Ca^{2+} release or in SR Ca^{2+} leak. This in turn, could contribute to or counteract the mechanical recovery respectively. Acidosis-induced inhibition of PP1 could maintain the phosphorylation (activation) of CaMKII and of the different CaMKII substrates that are also targets of PP1. Dashed lines indicate mechanisms that are currently not fully established. Grey lines indicate inhibitory effects and black lines indicate stimulatory effects of acidosis. The line width indicates the relative contribution to the mechanical recovery of the different mechanisms triggered by acidosis.

shown that phosphorylation of the Thr^{17} site of PLN transiently increased at the onset of acidosis. Inhibition of this phosphorylation by 1 μM KN-93 produced a significant decrease in the 'early' recovery of relaxation observed within the first 5 min of acidosis, subsequent to the initial impairment produced by acidosis. As previously suggested, this phosphorylation would provide a mechanism to overcome the direct depressant effect of acidosis on SERCA2a [10,13]. The close temporal association of PLN phosphorylation with the relaxation recovery may explain the observation that the recovery of relaxation occurs substantially during the first 5 min of acidosis. In contrast to the important role of Thr^{17} phosphorylation in the relaxation recovery, the phosphorylation of this site could not be associated in these experiments with a significant improvement of the contractile recovery. The decrease in Thr^{17} phosphorylation produced by 1 μM KN-93 failed to affect the contractile recovery [24]. These results suggest that CaMKII-dependent phosphorylation of proteins other than PLN may play a role in the contractile recovery during acidosis, at least in the absence of PLN phosphorylation, i.e. 5 μM KN-93, a concentration of the CaMKII inhibitor that produced a decrease in Thr^{17} phosphorylation and relaxation recovery similar to 1 μM KN-93, significantly inhibited the contractile recovery. Furthermore, the Thr^{17} site became dephosphorylated after 5 min of acidosis, which makes it an unlikely candidate to account for the modest but significant contractile recovery that occurs beyond 5 min of acidosis. However this recovery was still blocked by 5 μM KN-93 [24]. The finding that the concentration of the CaMKII inhibitor required to completely

inhibit the contractile recovery (5 μM) was higher than the concentration that inhibits Thr^{17} phosphorylation and the relaxation recovery (1 μM) suggests, in addition, functional compartmentalization of the enzyme.

7.2. Possible role of CaMKII-dependent phosphorylation of proteins other than PLN

Putative candidates among the proteins other than PLN possibly involved in the contractile recovery during acidosis are RyR2, L-type Ca^{2+} channel and NHE. The activity of the RyR2 is tightly regulated by several factors, including the CaMKII phosphorylation of different residues [69,70]. It is therefore likely that acidosis increases CaMKII-dependent RyR2 phosphorylation, due to enhanced CaMKII activity and the acidosis-induced inhibition of PP1, a RyR2 dephosphorylating phosphatase. Although the functional consequences of RyR2 phosphorylation are controversial, there is strong evidence that phosphorylation of RyR2 increases channel activity during diastole (as SR Ca^{2+} leak or Ca^{2+} sparks) and during activation (enhancing fractional Ca^{2+} release, for a given SR Ca^{2+} content and I_{Ca} trigger) [62,71,72]. Thus, an increase in RyR2 phosphorylation produced by acidosis might either promote SR Ca^{2+} leak or enhance SR Ca^{2+} release. In the former case, RyR2 phosphorylation would contribute to a decrease in SR Ca^{2+} content, opposing the mechanical recovery. The possibility of a leak of Ca^{2+} from the SR is not supported by the experimental evidence, which indicates increased SR Ca^{2+} content during acidosis [20]. In the latter case, the enhanced SR Ca^{2+} release that may result from RyR2 phosphorylation, would favor the mechanical recovery. However, experiments from Eisner's group [41,73,74] clearly demonstrate that in conditions where the SR Ca^{2+} content is not controlled, factors that alter the sensitivity of RyR2 to trigger Ca^{2+} will have only transitory effects on the amplitude of Ca^{2+} transients. This is because the increase in Ca^{2+} transient amplitude decreases Ca^{2+} influx via the L-type Ca^{2+} channel and increases Ca^{2+} efflux via NCX. Ultimately this would decrease SR Ca^{2+} content, counteracting the initial effect of RyR2 phosphorylation. Such a decrease in SR Ca^{2+} content would not occur however, in the context of increased phosphorylation PLN at the Thr^{17} site which would promote maintenance of the SR Ca^{2+} load. Unfortunately no experimental data have yet been produced to examine the effect of acidosis on RyR2 phosphorylation and its possible functional consequences.

The regulation of NHE and I_{Ca} by CaMKII (as discussed above) might also contribute to the CaMKII dependence of the mechanical recovery, since activation of these mechanisms would augment SR Ca^{2+} load. In addition activation of NHE may also mediate an enhancement of myofilament Ca^{2+} responsiveness through the recovery of pH_i. Although as discussed above, the phosphorylation of NHE by CaMKII still remains controversial [26,59], a CaMKII-dependent increase in I_{Ca} has been reported by different laboratories [26,62].

In summary, a review of available experimental evidence supports the view that CaMKII plays an essential role in the sequence of events underpinning the mechanical recovery from acidosis: phosphorylation of the Thr^{17} site of PLN appears to be primarily responsible for the relaxation recovery that occurs during acidosis, whereas phosphorylation of other proteins appears to be involved in the contractile recovery. This sequence of events is presented schematically in Fig. 2.

Of interest, an increase in the phosphorylation of Thr^{17} site of PLN has been observed in the *stunned heart* at the beginning of reperfusion (1–3 min) [75,76]. This increased phosphorylation appears to be the consequence of two associated phenomena coincident at the onset of reperfusion i.e. the Ca^{2+} overload triggered by reperfusion, that would activate CaMKII, and the intracellular acidosis, persisting from the preceding ischemia [75,76], that would in addition inhibit PP1 [55]. As in the situation of an acid load, in reperfusion it has been shown that CaMKII-dependent phosphorylation of Thr^{17} site of PLN, promotes SERCA2a activity and provides a mechanism that favors the mechanical and Ca^{2+} transient recoveries [76,77]. This tends to counteract the detrimental effect of Ca^{2+} overload through an early clearing of excessive Ca^{2+} from the cytosol. In this context it is important to note that, in contrast to the beneficial effect of CaMKII in the *stunned heart* mediated by the phosphorylation of Thr^{17} of PLN, activation of CaMKII has been recently described as a major cause of apoptosis during the irreversible ischemia/reperfusion injury [78,79]. Moreover, post-ischemic reperfusion and prolonged acidosis are known to trigger arrhythmias, a phenomenon in which sustained CaMKII activation may play a significant role [80].

8. Conclusions

CaMKII-dependent phosphorylation appears to be a crucial mechanism essential for mechanical recovery during acidosis. The finding that this recovery is absent in the presence of CaMKII inhibition, provides strong evidence that other mechanisms triggered by acidosis, including the rise in diastolic Ca^{2+} and the inhibition of RyR2, do not account for the increases in SR Ca^{2+} load and Ca^{2+} transient amplitude which characterize the contractile recovery during acidosis. These mechanisms seem to be required however for the activation of CaMKII, which then acts as an essential mediator of mechanical recovery. Although in this scenario, CaMKII-dependent phosphorylation of PLN appears to be primarily responsible for the enhancement of relaxation and the increase in SR Ca^{2+} load, CaMKII-dependent phosphorylation of proteins other than PLN also appears to play a role in the contractile recovery from acidosis.

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