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Original article

Role of CaMKII and ROS in rapid pacing-induced apoptosis

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ABSTRACT

Tachycardia promotes cell death and cardiac remodeling, leading to congestive heart failure. However, the underlying mechanism of tachycardia- or rapid pacing (RP)-induced cell death remains unknown. Myocyte loss by apoptosis is recognized as a critical factor in the progression to heart failure and simulation of tachycardia by RP has been shown to increase the intracellular levels of at least two potentially proapoptotic molecules, Ca^{2+} and reactive oxygen species (ROS). However, whether these molecules mediate tachycardia- or RP-induced cell death has yet to be determined. The aim of this study was to examine the subcellular mechanisms underlying RP-induced apoptosis. For this purpose rat ventricular myocytes were maintained quiescent or paced at 0.5, 5 and 8 Hz for 1 hr. RP at 5 and 8 Hz decreased myocyte viability by 58 ± 3% and 75 ± 6% (n = 24), respectively, compared to cells maintained at 0.5 Hz, and increased caspase-3 activity and Bax/Bcl-2 ratio, indicative of apoptosis. RP-induced cell death and apoptosis were prevented when pacing protocols were conducted in the presence of either the ROS scavenger, MPG, or nifedipine to reduce Ca^{2+} entry or the CaMKII inhibitors, KN93 and AIP. Consistently, myocytes from transgenic mice expressing a CaMKII inhibitory peptide (AC3-I) were protected against RP-induced cell death. Interestingly, tetracaine and carvedilol used to reduce ryanodine receptor (RyR) diastolic Ca^{2+} release, and ruthenium red used to prevent Ca^{2+} entry into the mitochondria prevented RP-induced cell death, whereas PI3K inhibition with Wortmannin exacerbated pacing-induced cell mortality. We conclude that CaMKII activation and ROS production are involved in RP-induced apoptosis. Particularly, our results suggest that CaMKII-dependent posttranslational modifications of the cardiac ryanodine receptor (RyR) leading to enhanced diastolic Ca^{2+} release and mitochondrial Ca^{2+} overload could be the underlying mechanism involved. We further show that RP simultaneously activates a protective cascade involving PI3K/AKT signaling which is however, insufficient to completely suppress apoptosis.

1. Introduction

Experimental and clinical evidence indicates that sustained tachycardia and rapid pacing (RP) promote cardiac remodeling leading to congestive heart failure [1–3]. At present, myocyte loss by apoptosis is recognized as a critical factor in the progression to heart failure and both tachycardia and RP have been shown to promote apoptosis [2,4]. Several lines of evidence indicate that the elevation of pacing rate increases the intracellular levels of at least two, potentially proapoptotic molecules, Ca^{2+} and reactive oxygen species (ROS) [5–7]. However, whether these molecules mediate tachycardia or RP-induced cell death is unclear.

Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) is a ubiquitous kinase that is canonically activated by the elevation of intracellular Ca^{2+} but more recently, its activity has also been shown to be modulated by ROS-dependent oxidation [8,9]. CaMKII is known to play an important physiologic role in the regulation of cardiac excitation–contraction coupling [10]. However, under pathological conditions, chronic activation of CaMKII has been shown be proapoptotic and detrimental [11]. Indeed, activation of CaMKII has been shown to be a common intermediate of diverse death stimuli that induce apoptosis in cardiac cells [12]. Consistent with these results, we have recently demonstrated that CaMKII activation is a critical step in the signaling cascade that leads to apoptosis in ischemia/reperfusion injury, ouabain toxicity, and sustained Angiotensin II (Ang II) stimulation [9,13,14].

When pacing frequency is increased, cytosolic Ca^{2+} transient amplitude is augmented, which may activate CaMKII [15]. This RP-induced CaMKII activation could further increase the Ca^{2+} transient amplitude due to phosphorylation of various Ca^{2+} handling proteins. If CaMKII activation is sustained, it could lead to deleterious Ca^{2+} overload and sarcoplasmic reticulum (SR) Ca^{2+} leak that could trigger the apoptotic cascade. During sustained tachycardia or RP, ROS is also generated, which can also activate CaMKII, thus favoring the apoptotic process. ROS can also contribute to apoptosis independently of its effect on CaMKII activity. In the present study we tested the hypothesis that RP promotes CaMKII activation and ROS production, which leads to the activation of the apoptotic cascade and cardiac cell death.

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RP has also been shown to trigger the activation of the prosurvival pathway, involving the PI3K/AKT cascade [4]. Thus, we also investigated whether the activation of this cascade protects the heart from RP-induced apoptosis.

The results presented herein, using pharmacological tools and genetic manipulation, show that RP-induced apoptosis in cardiac cells involves Ca^{2+}-dependent CaMKII activation and ROS production. We further show that RP-induced CaMKII-dependent cell death and apoptosis are, at least in part, counterbalanced by the simultaneous activation of prosurvival PI3K/AKT signaling. Importantly, we show that the cardiac ryanodine receptor (RyR2) plays a critical role in RP-induced cell death, and that agents that reduce RyR2 open probability (RyR2 stabilizers) are effective in preventing the deleterious effects of RP.

2. Methods

2.1. Myocyte isolation and culture

All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No.85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of La Plata University.

Wistar rats (200–300 g) or transgenic mice with cardiomyocyte-delimited transgenic expression of either a CaMKII inhibitory peptide (AC3-I) or a scrambled control peptide (AC3-C) or mutant mice where the CaMKII-dependent phosphorylation site on the RyR2 (site Ser 2814) is mutated to alanine (Ser 2814a) were anesthetized by intra-abdominal injection of sodium pentobarbitone (35 mg (kg body weight) − 1). Myocytes were isolated by enzymatic digestion [13] and kept in a HEPES buffered solution at room temperature (20–22 °C), until use. Unless otherwise specified, experiments were performed at 37 °C. Myocytes in HEPES buffer were plated at a density of ~2×10^4 rod-shaped cells/ml into culture dishes for 1 h to allow cell attachment. The media was then changed for a fresh one with or without the presence of pharmacological compounds according to the experiments performed (see Results). Culture dishes were then supplemented with carbon electrodes used to continuously pace cells at 0.5, 5 and 8 Hz for 1 hr. After the 1 hr stimulation period cells were photographed to assess viability.

2.2. Assessment of cell viability

Cells were evaluated morphologically, being classified as viable or nonviable according to their length-to-width ratio (>3 were considered viable) [13]. From each culture, which was considered as an n equal to 1, at least 8 photographs per group were taken to count and classify the cells. Necrosis was assessed by trypan blue staining.

2.3. Tissue preparation

Langendorff perfusion: Isolated rat hearts were perfused according to the Langendorff technique at constant temperature (37 °C) and coronary flow (12–14 ml/min) as previously described [14]. Experimental protocol: After stabilization, hearts were paced at 0.5, 5 or 8 Hz for 1 hr. For kinase or ROS production inhibition, hearts were treated with inhibitors during the stabilization period and for the entire pacing period. After pacing for 1 hr at the different pacing frequencies either in the absence or presence of inhibitors, hearts were freeze-clamped and stored at −80 °C for biochemical assays.

2.4. Western blot

For immunological detection, cardiac homogenates and cytosolic fractions were prepared from the pulverized ventricular tissue from Langendorff perfused rat hearts as previously described [14]. Proteins were electrophoresed and transferred to PVDF membranes as previously described [14]. Blots were probed with antibodies raised against caspase-3, phospho-CaMKII (Chemicon International), Bcl-2, Bax (Santa Cruz Biotechnology), GAPDH, and phospho-AKT. Immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Immobilon Western Millipore) using a Chemidoc Imaging System. The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software (NIH). Apoptosis was determined by immunoblot of the pro- and anti-apoptotic proteins Bax and Bcl-2, respectively (Santa Cruz Biotechnology). Caspase-3 activity was also determined by western blot.

2.5. Intracellular Ca^{2+} and ROS production measurements

For intracellular Ca^{2+} measurements isolated myocytes were loaded with 10 μM Fura-2 AM [9]. Cells were placed on the stage of an inverted microscope (Nikon Diaphot 200) adapted for epifluorescence by an IonOptix hardware. Cells were continuously superfused with HEPES buffered solution (pH 7.4) at a constant flow of 1 ml/min and field stimulated at 0.5, 5 and 8 Hz via two platinum electrodes on either side of the bath. The ratio of the Fura-2 AM fluorescence obtained after exciting the dye at 340 and 380 nm was taken as an index of Ca^{2+}i. Cells loaded with 10 μmol/l Fluo-3 were used to visualize Ca^{2+} waves using a Zeiss 410 inverted confocal microscope (LSM Tech, Pennsylvania, USA). Confocal images of Ca^{2+} waves were taken in the line scan mode [16]. Cells were excited with the 488 nm line of an argon laser and fluorescence was collected at >515 nm. Each image consisted of 512 line scans obtained at 4 ms intervals. The number of waves in each frame were counted by visual inspection and normalized to SR Ca^{2+} content obtained from the amplitude of the caffeine (20 mM) induced Ca^{2+} transient. Waves were acquired for 60 s after 1 hr stimulation at 0.5, 5 or 8 Hz, either in the presence or absence of KN93 (1–2.5 μmol/l), KN92 (2.5 μmol/l) or AIP (1 μM). Intracellular ROS production was measured on a Zeiss 410 microscope using rat ventricular myocytes loaded with 5 μM of 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCF DA, Invitrogen/Molecular Probes) for 30 min at 37 °C. CM-H2DCF DA (DCF) was excited at 488 nm and the emitted fluorescence was recorded at 510–560 nm. As DCF can produce artifactual signal amplification upon continuous light exposure, cells were imaged at extremely low intensity and images were taken every 180 s to minimize light exposure during the duration of the experiment. H_2O_2 was used as a control for ROS detection capacity.

2.6. Statistical analysis

Unpaired Student *t* test or one-way ANOVA was used for statistical comparisons when appropriate. Data are expressed as means ± SEM. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Rapid pacing reduces myocyte viability and promotes apoptosis

The effect of electrical stimulation at 0.5, 5 and 8 Hz for 1 hr on cell viability was tested in isolated rat ventricular myocytes. Fig. 1A depicts typical images and average results showing that pacing myocytes at 5 and 8 Hz produced a 58 ± 3% and 75 ± 6% (n = 24) reduction in cell viability compared to those maintained at 0.5 Hz. In contrast, pacing at 0.5 Hz did not affect cell viability compared to quiescent cells (64 ± 3 vs. 62 ± 2% viability for quiescent and 0.5 Hz paced cells, respectively). Similar results were obtained in control experiments where electrical stimulation was maintained at 0.5, 5 and 8 Hz for 24 hr (not shown). Since the activation of caspases is a critical step in apoptosis, caspase-3 activity was determined in samples from Langendorff perfused rat hearts paced for 1 hr at the frequencies indicated above. Fig. 1B shows typical blots and average results indicating

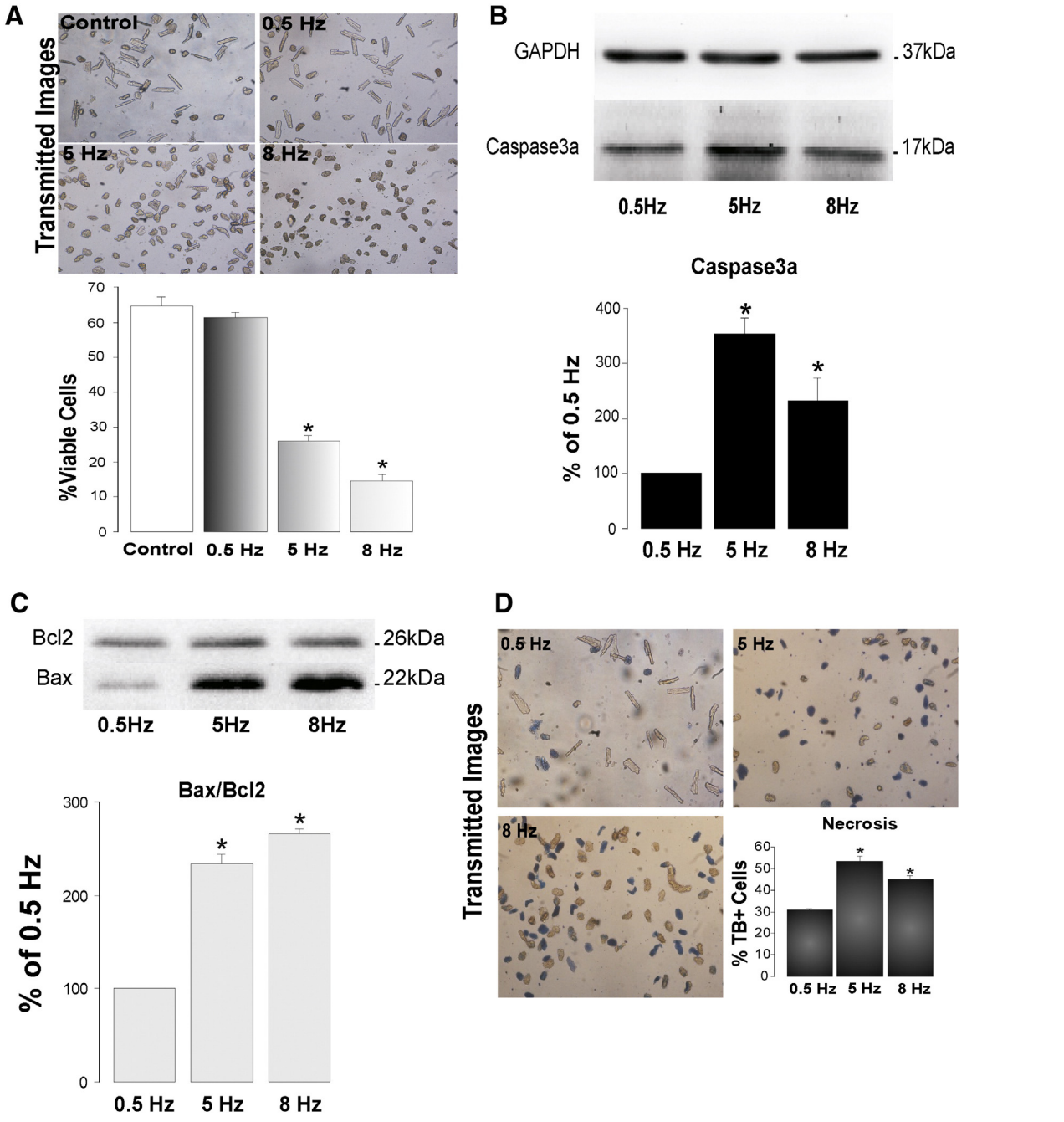

Fig. 1. Rapid pacing reduces cell viability and promotes apoptosis in rat cardiac myocytes. A: Representative bright field images and average results showing that the number of viable cells was significantly reduced after 1 hr of RP at 5 and 8 Hz (n = 24). B: Representative western blots and overall results indicating that RP significantly increases caspase-3 activity (n = 5). C: Representative blots showing that RP increased the expression of the proapoptotic protein Bax. The bar graph below depicts the overall results of these experiments expressed as the ratio between Bax and Bcl-2 (n = 5). D: Representative photographs and average results showing that RP increased the number on trypan blue positive cells, indicative of necrosis. Data are expressed as means ± S.E.M. *p < 0.05, vs. 0.5 Hz.

that pacing cells at 5 and 8 Hz significantly increase caspase-3 activity compared to those maintained at 0.5 Hz (n = 4 from 4 hearts). Similarly, Fig. 1C depicts typical blots and average data further demonstrating that the reduction in cell viability induced by rapid pacing was also associated with an increase in the ratio between pro- and anti-apoptotic proteins Bax and Bcl-2 (bax/bcl-2) (n = 4 from 4 hearts). The number of trypan blue positive cells was also assessed to evaluate necrosis. As shown in Fig. 1D, RP significantly increased the number of trypan blue positive cell from 30 ± 2 % at 0.5 Hz to 54 ± 3 % and 47 ± 3% at 5 and 8 Hz (n = 9), respectively, indicating that necrosis is also in part responsible for RP-induced cell death.

3.2. CaMKII mediates rapid pacing-induced cell death

We and others have shown that CaMKII is a common intermediary of diverse apoptosis-inducing insults [9,12–14]. To evaluate the role of CaMKII in rapid pacing-induced cell death and apoptotic signaling, we assessed whether: 1) RP leads to production of signals for CaMKII activation and to activation of the kinase, 2) RP-induced cell death and apoptosis can be prevented by pharmacological inhibition of CaMKII with KN-93, AIP or using the inactive KN93 analog, KN92 and 3) transgenic mouse myocytes expressing the CaMKII inhibitory peptide AC3-I are protected from RP-induced cell death.

Fig. 2 shows the effect of RP on the Ca^{2+} transient (CaiT), reactive oxygen species (ROS) production and CaMKII activity (p-CaMKII). Panel A depicts overall data showing that RP significantly increases both diastolic and systolic Ca^{2+} as well as ROS production compared to 0.5 Hz. These results indicate that RP can produce the stimuli for CaMKII activation (Ca^{2+} and ROS). Consistently, the typical blots and average data shown in panel B indicate, as expected, that RP increases CaMKII activity (n = 5). Fig. 3A depicts typical images and average results showing that pacing myocytes in the presence of 2.5 μM of the CaMKII inhibitor, KN93, did not result in cell death. As shown in panel B, the protective effect of KN93 is at least in part due to its ability to reduce apoptosis as indicated by the failure of RP to increase caspase 3 activity and Bax/Bcl-2 ratio in the presence of KN93. In addition, control experiments show that KN93 can prevent RP-induced increase in CaMKII activity. To discard possible nonspecific effects of KN93, the effect of RP on cell viability was also assessed in the presence of 1 μM of the more specific CaMKII-inhibitory peptide, AIP or in the presence of the inactive KN93 analog KN92. The bar graph on the right of Fig. 3A shows that similar to the results obtained using KN93, AIP completely prevented RP-induced cell death whereas KN92 failed to protect cells from RP-induced injury. Moreover, a lower dose of KN93 (1 μM) was also able to prevent RP-induced cell mortality. To further confirm the involvement of CaMKII in RP-induced cell death, Fig. 3C shows that transgenic myocytes expressing the CaMKII inhibitory peptide (AC3-I) were protected against the deleterious effects of RP when compared with myocytes expressing the scrambled control peptide (AC3-C).

3.3. CaMKII-induced ROS production underlies RP-induced cell death

To evaluate whether RP-induced ROS production contributes to CaMKII activation or whether ROS production is downstream of CaMKII, we assessed CaMKII activity in the presence of the ROS scavenger, MPG, or monitored RP-induced ROS production in the presence of KN93. Fig. 4A shows typical blots and average results indicating that RP-induced CaMKII activation is maintained in the presence of MPG. In contrast, as observed in the typical fluorescence images and average results presented in Fig. 4B, RP failed to enhance ROS production in the presence of KN93. Furthermore, RP failed to increase ROS production in the presence of the more specific CaMKII inhibitor, AIP. In contrast, RP significantly increased ROS production in the presence of the inactive analog KN92. Moreover, RP-induced ROS production was abrogated in myocytes from AC3-I mice whereas RP significantly increased ROS in AC3-C myocytes (131 ± 3% increase in ROS fluorescence at 5 Hz in AC3-C (n = 4) vs. 97 ± 10% increase in ROS fluorescence at 5 Hz in AC3-I myocytes (n = 4)); these results suggest that RP-induced ROS production is not required for CaMKII activation, whereas CaMKII activation is necessary for RP-induced ROS production. To assess whether CaMKII-dependent ROS production underlies RP-induced cell death and apoptosis, we examined these processes in the presence of MPG. Figs. 5A and B depicts typical transmitted images and average results showing that MPG prevents RP-induced decrease in cell viability and reduces the increase in apoptotic indexes, caspase-3 activity and Bcl-2/Bax ratio.

3.4. Mechanisms underlying CaMKII-mediated RP-induced cell death

Ryanodine receptors type 2 (RyR2) are crucial for physiologic myocyte intracellular Ca^{2+} handling. Nevertheless, abnormal Ca^{2+} handling and Ca^{2+} leak via the RyR2 have recently been shown by us and others to be key determinants of cardiomyocyte apoptosis under different pathological conditions [17–19]. Ca^{2+} leak from the SR results in spontaneous Ca^{2+} waves. Thus, using confocal imaging we assessed Ca^{2+} wave frequency normalized by SR Ca^{2+} load at each frequency as an index of Ca^{2+} leak [20]. Consistent with previous findings in the rat heart, increasing pacing frequency from 0.5 Hz to 5 Hz and 8 Hz did not significantly affect SR Ca^{2+} content [21]. Nevertheless, there was a tendency for KN93 to increase SR content which however did not reach statistical significance. Fig. 6A shows representative fluorescence images and average data demonstrating that RP increases wave frequency. Fig. 6A further shows that the RP-induced increase in Ca^{2+} wave frequency was prevented by KN93, indicating that CaMKII underlies these events. We further tested the possible involvement of RyR2 in RP-induced cell death by using structurally different pharmacological compounds known to modulate RyR2 open probability or to suppress arrhythmias. Fig. 6B depicts average results showing that treatment with the RyR2 stabilizers, tetracaine, carvedilol, or the minimally beta blocking carvedilol analog, VK-II-86, prevented RP-induced cell death. These results suggest that the RyR2, possibly through a RP-induced enhancement in Ca^{2+} leak, is functionally involved in RP-induced cell death. RyR2 function can be modulated at least in part by posttranslational modifications, including phosphorylation and/or oxidation. Using myocytes from transgenic mice lacking the RyR2 CaMKII-dependent phosphorylation site, serine 2814 (2812a), we examined the role of this phosphorylation on RP-induced cell death. Fig. 6C shows that RP similarly decreased cell viability in both 2814a myocytes and WT controls, indicating

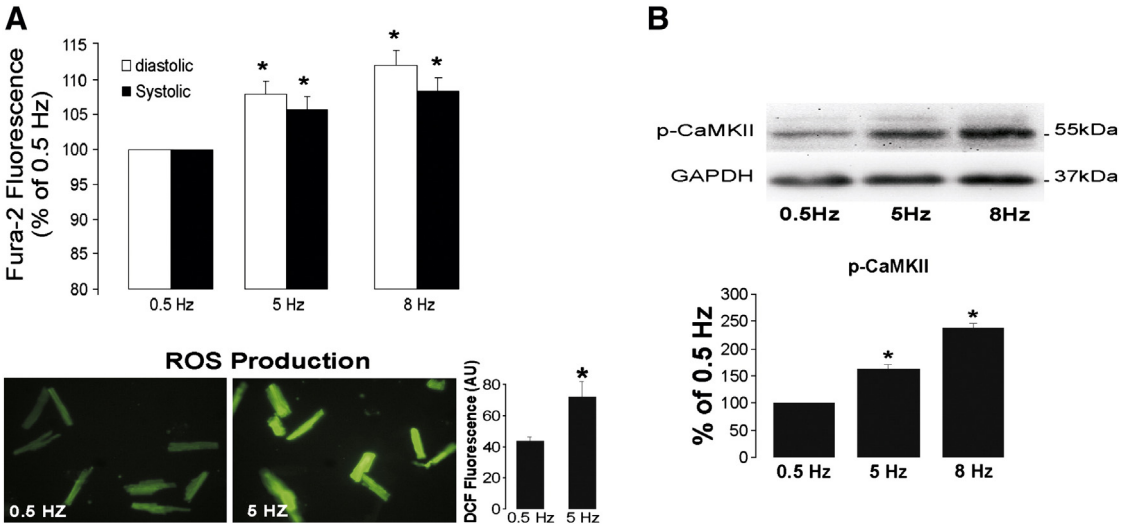

Fig. 2. Rapid pacing increases intracellular Ca^{2+} transient, enhances ROS production and activates CaMKII. Effect of RP on the intracellular Ca^{2+} transient (Fura 2 fluorescence), ROS production (DCF DA fluorescence) and CaMKII activity (p-CaMKII). A: The bar graph shows that rapid pacing significantly increases both diastolic and systolic Ca^{2+} (n = 6). The typical fluorescence images below show that 1 hr of RP significantly increases ROS production (n = 4). B: Typical western blot showing that RP at 5 and 8 Hz significantly increases CaMKII activity (n = 5).

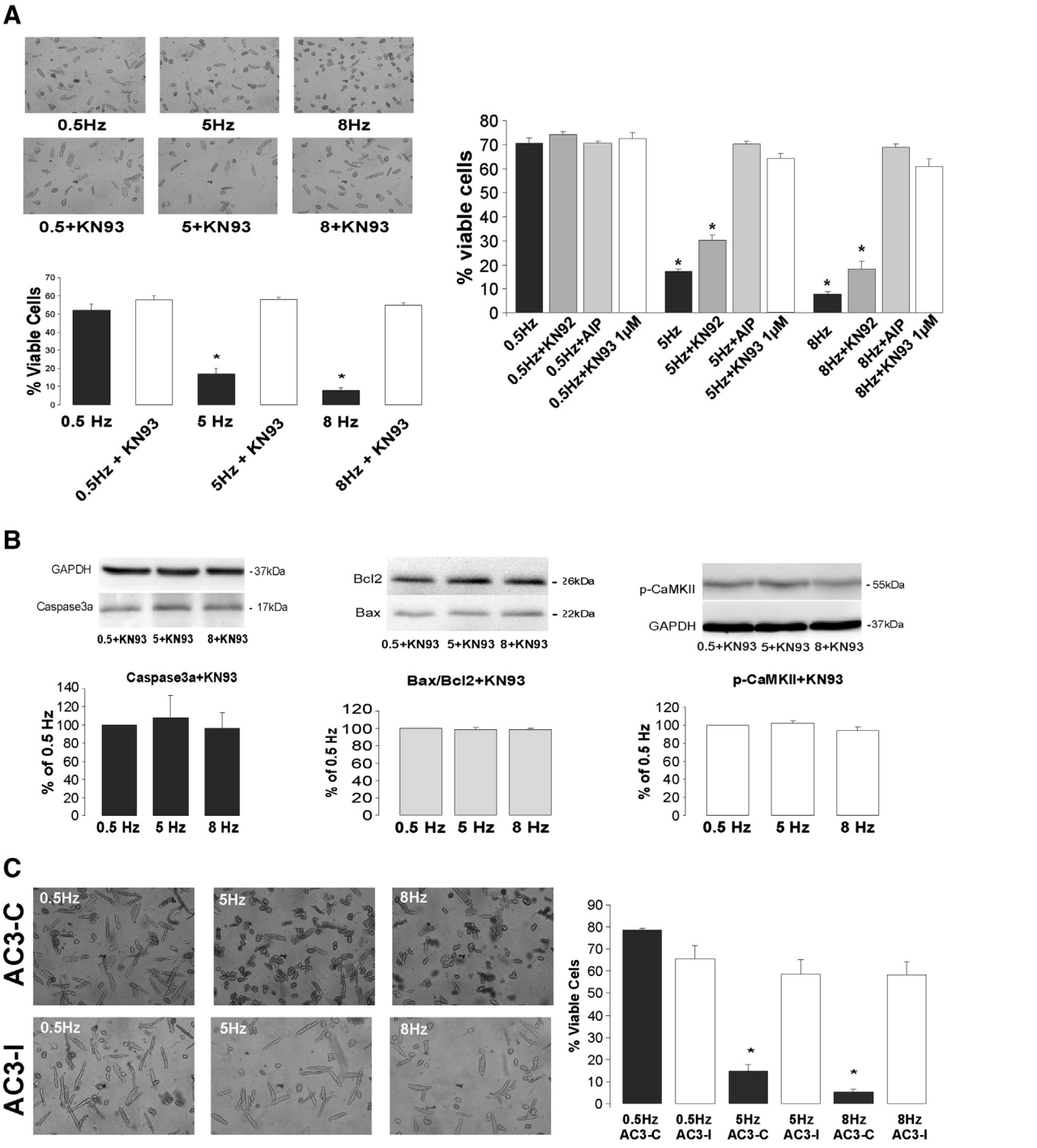

Fig. 3. CaMKII activation mediates rapid pacing-induced cell death and apoptosis. Panel A: Representative transmitted images of myocytes paced at low (0.5 Hz) and high (5 and 8 Hz) frequencies either in the absence or in the presence of 2.5 μM of the CaMKII inhibitor KN93 (KN93). These images and the bar graphs below clearly show that KN93 prevents RP-induced cell death (n = 9). The bar graph on the right shows overall results of the effect of RP on myocyte viability either in the absence or the presence of 2.5 μM KN92 (n = 5), 1 μM AIP (n = 5) or 1 μM KN93 (n = 5). *$p < 0.05$, vs. 0.5 Hz. Panel B depicts representative blots and overall results showing that KN93 prevents the increase in caspase 3 activity, Bax/Bcl-2 ratio and CaMKII activity induced by RP (n = 4). Panel C shows typical transmitted images and overall results of the effect of RP on the viability of cells isolated from transgenic mice overexpressing a CaMKII inhibitory peptide (AC3-I) or from myocytes expressing the scrambled control peptide (AC3-C) (n = 4 for each group). AC3-I expressing myocytes were protected from RP-induced cell death. *$p < 0.05$, vs. 0.5 Hz.

that CaMKII-dependent phosphorylation of the RyR2 was not involved. Interestingly, 2814a and WT myocytes were protected from RP-induced cell death by pre-treatment with the ROS scavenger MPG (Fig. 6C). To assess whether SR Ca^{2+} leak promotes mitochondrial Ca^{2+} overload, resulting in the opening of the mitochondrial permeability transition pore and activation of the apoptotic cascade, is the underlying mechanism of RP-induced cell death, we inhibited mitochondrial Ca^{2+} uptake via the uniporter with 5 μM ruthenium red and the opening of the permeability transition pore with 10 μM bongkrekic acid. Fig. 6D shows that both these interventions were able to prevent RP-induced reduction in cell viability.

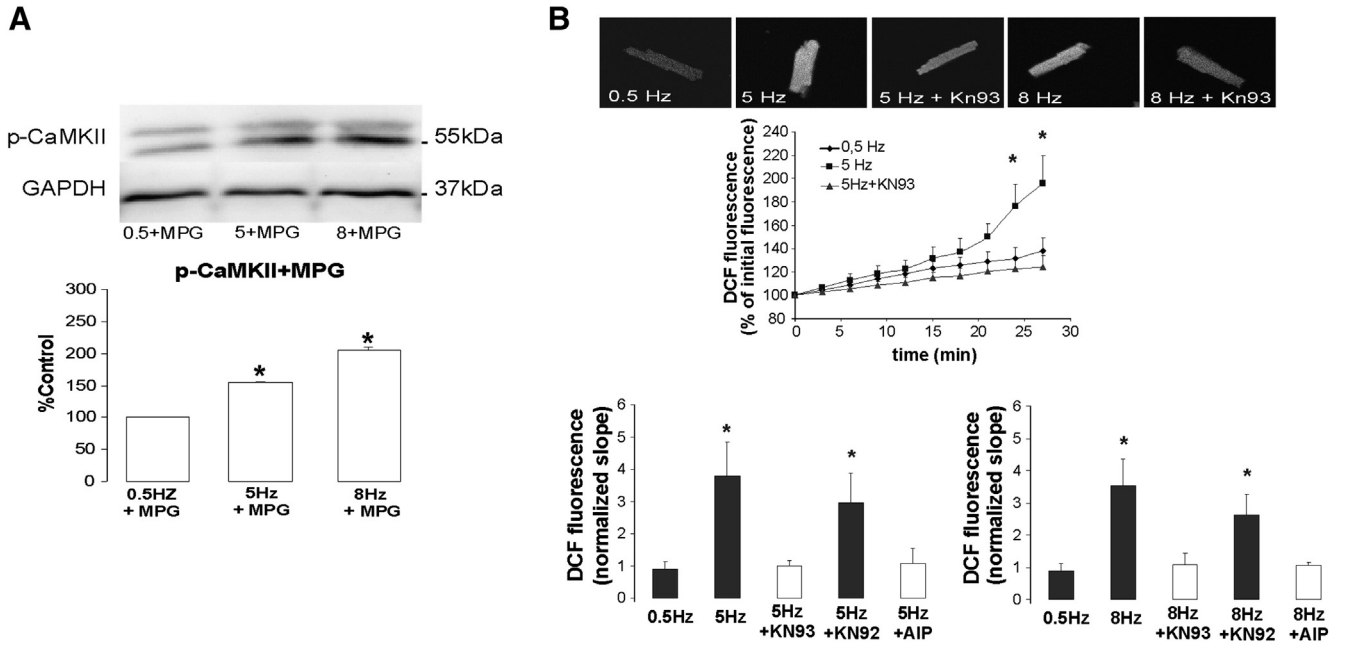

Fig. 4. Rapid pacing-induced ROS production requires CaMKII activation. A: Representative blots and overall results showing the effect of RP on CaMKII activity in the presence of 1 μM of the ROS scavenger, MPG (n = 5). B: Representative fluorescence images of the effect of RP on DCF fluorescence in the absence and presence of 2.5 μM KN93. The overall results below show the time course of RP-induced ROS production in the absence and presence of KN93 and the normalized DCF slope in the absence and presence of KN93, KN92 (n = 7) and AIP (n = 6). KN93 and AIP significantly reduced the 5- and 8 Hz-induced increase in DCF fluorescence whereas ROS significantly increased at 5 and 8 Hz in the presence of KN92.

3.5. Inhibiting PI3/AKT signaling exacerbates the deleterious effects of rapid pacing on myocyte viability

RP has been shown to promote early activation of the prosurvival kinase, AKT [4]. We have recently demonstrated that CaMKII mediates ouabain-induced apoptosis and that this process is counterbalanced by AKT activation which serves to protect from larger damage [13]. To examine whether PI3K/AKT pathway also serves a protective role in RP-induced cell death we paced cells in the absence and presence of the PI3K inhibitor Wortmannin. Fig. 7 shows the effect of RP on AKT activity and cell viability. RP significantly increased AKT activity, and inhibiting PI3K/AKT signaling significantly augmented the number of dead cells compared to RP alone. These results confirm that RP in addition to the activation of an apoptotic cascade, triggered by CaMKII activation, simultaneously activates a prosurvival cascade involving PI3K, which is, however, insufficient to completely repress apoptosis. Control experiments showed that Wortmannin did not per se affect cell viability in quiescent cells (not shown).

4. Discussion

Sustained tachycardia in patients and in experimental rapid pacing animal models has been shown to promote mechanical and electrical remodeling leading to congestive heart failure [1–3]. Several reports have proposed myocyte apoptosis as a critical component of this adverse remodeling [4,22,23]. However, the subcellular mechanisms underlying myocyte cell death at supraphysiological heart rates have yet to be determined.

Using a cellular model of RP, Kuramochi et al. showed that RP-induced cell death is an early event that is associated with the activation of distinct stress kinases (Erk, AKT, JNK and p38MAPK) [4]. However, the study of Kuramochi et al. did not provide direct evidence supporting a causal role for these kinases in RP-induced cell death. Hanna et al. also found early induction of apoptosis in a canine model of RP. However, activation of MAPK signaling was not observed until 5 weeks after the onset of RP [23]. These results suggest that signaling events, other than MAPK's, are required for early RP-induced cell death and apoptosis.

RP is known to increase intracellular Ca^{2+} levels [5,24] and sustained Ca^{2+}i elevations have been shown to promote cardiomyocyte cell death [25]. Further, RP has been shown to enhance oxidative stress and experimental evidence demonstrates that antioxidants can inhibit cardiomyocyte apoptosis in a RP-induced heart failure model [26]. These results suggest that RP-induced Ca^{2+} and ROS elevation could underlie RP-induced apoptosis.

Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) is a ubiquitous threonine/serine kinase that is canonically activated by the elevation of intracellular Ca^{2+}. More recently, CaMKII activity has also been shown to be modulated by ROS-dependent oxidation [8,9] and sustained activation of CaMKII has been shown to be proapoptotic [11]. Indeed, experimental evidence from our laboratory demonstrates that CaMKII activation is a critical event in the signaling cascade that leads to apoptosis in ischemia/reperfusion injury, ouabain toxicity and under sustained Angiotensin II (Ang II) stimulation [9,13,14]. These results suggest that CaMKII could be the target for the collective increase in Ca^{2+}i and ROS during RP which could serve to prolong the activity of the kinase, favoring the apoptotic process. Thus, in the present study we tested the hypothesis that RP augments Ca^{2+} and ROS, resulting in CaMKII activation which promotes cardiac cell apoptosis.

The results presented herein show for the first time that CaMKII mediates RP-induced cardiomyocyte apoptosis and we provide evidence suggesting that the underlying mechanism for RP-induced cell death may involve CaMKII-dependent ROS production leading, at least in part, to posttranslational modification of the RyR2 (oxidation), known to enhance diastolic Ca^{2+} release. This diastolic Ca^{2+} release as previously reported by us and others, would result in mitochondrial Ca^{2+} elevation and activation of the apoptotic cascade [18,19]. Our results further show that RP-induced cell death can be prevented by agents that reduce RyR2 open probability, raising the possibility of using RyR2 stabilizers as a therapeutic strategy to reduce the adverse remodeling associated with tachycardia and to prevent the progression of heart failure.

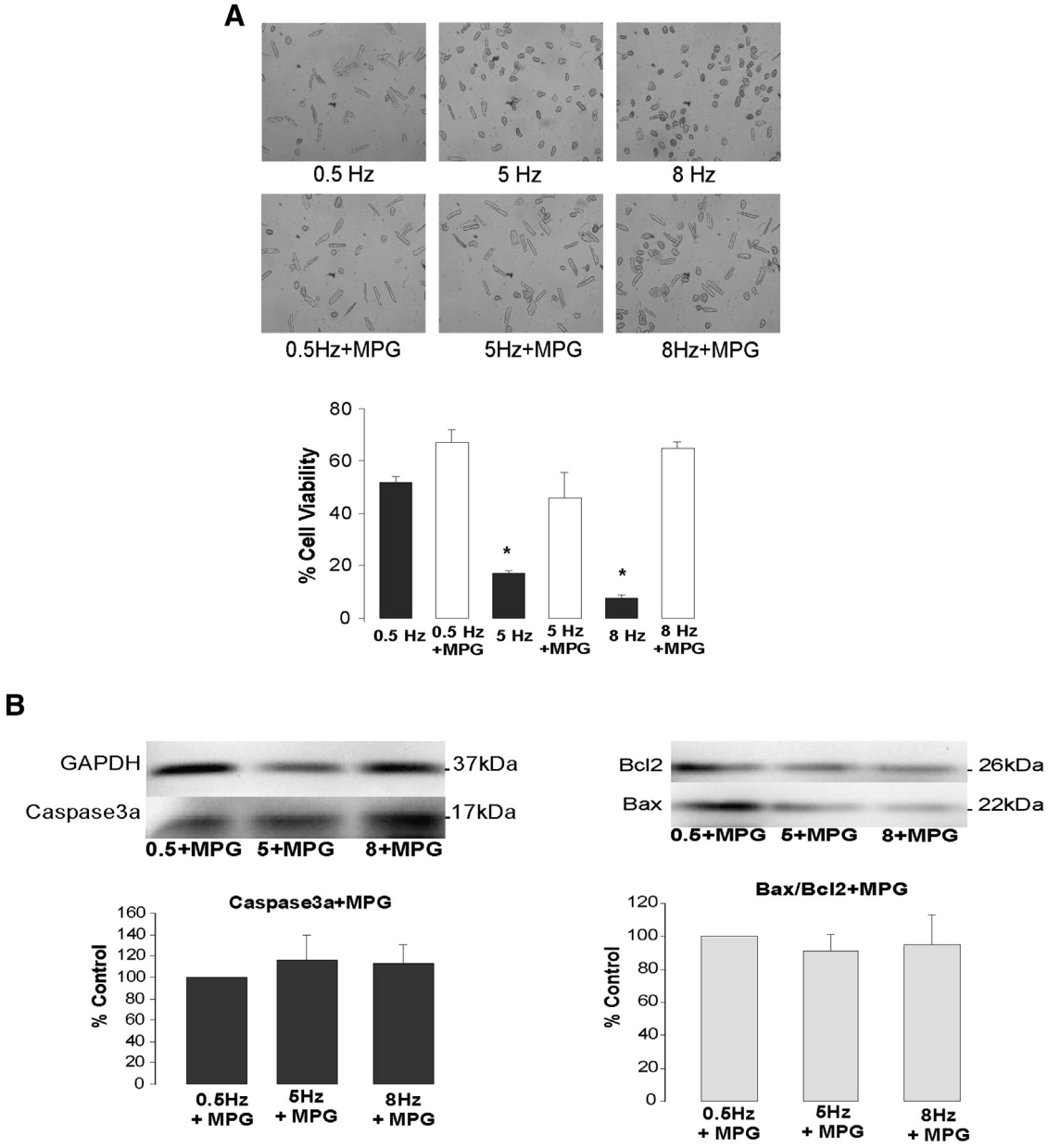

Fig. 5. Rapid pacing-induced ROS production mediates myocyte apoptosis. A: Representative transmitted images of myocytes paced at 0.5, 5 and 8 Hz either in the absence or in the presence of 1 μM of MPG. These images and the bar graphs below show that MPG treatment prevents RP-induced cell death (n = 5). Panel B depicts representative blots and overall results showing that MPG prevents the increase in caspase 3 activity and in the Bax/Bcl-2 ratio induced by RP (n = 4). *p < 0.05, vs. 0.5 Hz. Data are mean ± SEM from 4 independent experiments from 4 hearts.

4.1. CaMKII mediates RP-induced cell death

In accordance to previous findings, the present results clearly demonstrate that RP decreases myocyte viability by promoting apoptosis and necrosis. We showed that pacing cells at 5 and 8 Hz for 1 hr significantly reduced cell viability compared to those maintained at 0.5 Hz or maintained quiescent (control) and that this decrease in viability was associated with enhanced caspase-3 activity, one of the end effectors of the apoptotic cascade, and with increased Bax/Bcl-2 ratio which would suggest the involvement of mitochondria in the apoptotic process.

CaMKII has been shown to be a common intermediate through which diverse death-inducing stimuli trigger cardiomyocyte apoptosis [12] and RP is known to increase the two main modulators of CaMKII activity, Ca^{2+} and ROS [8,9,27]. Thus, we investigated the role played by CaMKII in RP-induced cell death. Confirming previous reports we observed that, under our experimental conditions, RP significantly increased diastolic and systolic Ca^{2+} as well as myocyte ROS production. Consistently, we found that RP increased the activity of CaMKII (p-CaMKII) and that KN93, a pharmacological inhibitor of CaMKII, could prevent the reduction in cell viability and the increase in the apoptotic indexes, Bax–Bcl-2 ratio and caspase-3 activity, induced by RP (Figs. 3A and B). Further confirming the involvement of CaMKII in RP-induced cell death we observed that RP induced cell mortality could also be prevented by the more specific CaMKII inhibitor, AIP, whereas, the KN93 inactive analog, KN92, did not suppress cell death. Moreover, transgenic mice expressing the CaMKII inhibitory peptide (AC3-I) were protected from the deleterious effects of high pacing frequencies (Fig. 3C). These findings from our combined studies of pharmacological inhibition and genetic manipulation discard potential unspecific effects

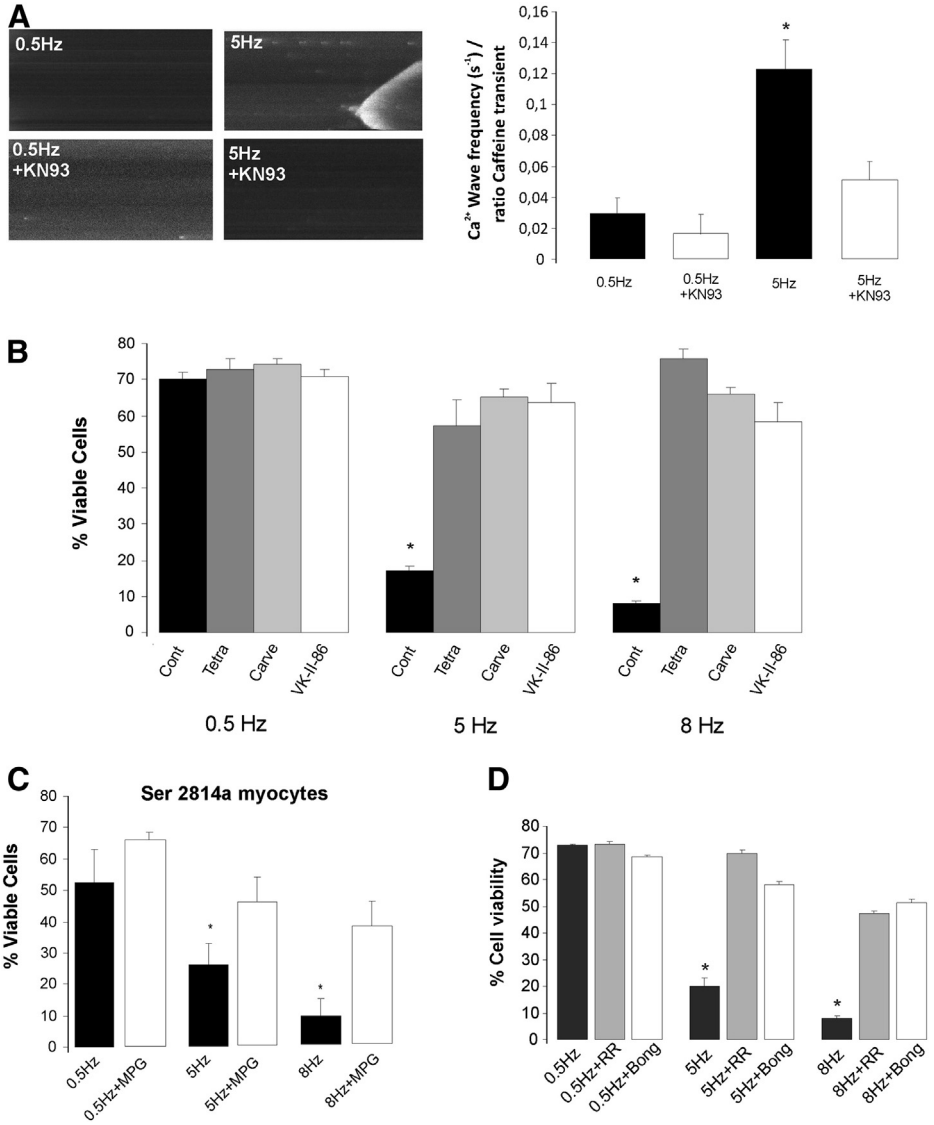

Fig. 6. Stabilizing the RyR2 prevents rapid pacing-induced cell death. A: Typical confocal line scan images and overall results showing that RP significantly increases Ca^{2+} wave occurrence and that this effect is inhibited by KN93. B: Overall results showing that RP-induced cell death can be prevented by the RyR2 stabilizers, tetracaine (25 μM), carvedilol (1 μM) or the carvedilol analog VK-II-86 (1 μM) (n = 6 to 8 per group). *p < 0.05, vs. 0.5 Hz. Panel C shows the effect of RP on cell viability of myocytes with the RyR2 phosphorylation site, serine 2814, mutated to alanine (2814a). RP significantly reduced 2814a myocyte viability and this reduction was prevented in the presence of the ROS scavenger, MPG (n = 6). *p < 0.05, vs. 0.5 Hz. D: Overall results showing that RP-induced cell death can be prevented by inhibiting the mitochondrial uniporter with 5 μM ruthenium red (RR) or by inhibiting the opening of the mitochondrial permeability transition pore with 10 μM bongkrekic acid (Bong) (n = 5 per group). *p < 0.05, vs. 0.5 Hz.

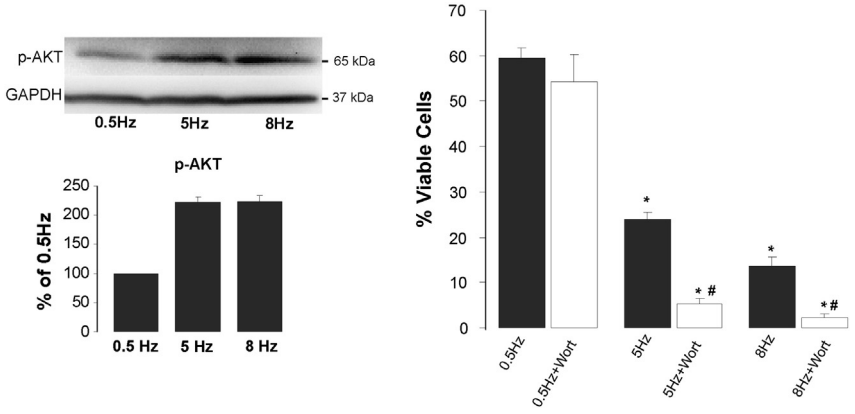

Fig. 7. Rapid pacing-induced reduction in cell viability is inhibited by PI3K/AKT activation. Representative blots and overall results showing that RP significantly increases p-AKT. The bar graph on the right shows average results (n = 8) of the effect of RP on cell viability either in the absence or the presence of 1 μM of the PI3K inhibitor Wortmannin (Wort). PI3K/AKT pathway inhibition significantly augmented the number of dead cells produced by RP alone. Data are expressed as means ± S.E.M. *p < 0.05, vs. 0.5 Hz; #p < 0.05, vs. 5 and 8 Hz without Wortmannin.

of KN93 and provide substantial evidence indicating that CaMKII is mechanistically involved in the deleterious effects of RP on cell viability.

We hypothesized that increased Ca^{2+}i and ROS production collectively contribute to the sustained activation of CaMKII during RP. However, we failed to observe a decrease in CaMKII activity when RP protocols were performed in the presence of the ROS scavenger, MPG (Fig. 4A). In contrast, we observed that RP-induced increase in CaMKII activity and cell death were prevented by the L-type Ca^{2+} channel blocker, nifedipine, used to inhibit enhanced Ca^{2+} entry produced during rapid pacing (not shown). These results indicate that during RP, CaMKII is activated by Ca^{2+} entry via the L-type Ca^{2+} channel and not by ROS-dependent oxidation. Similar to our results, increased ROS production by RP has been previously reported [6]. However, the source of ROS is still not completely defined. Several lines of evidence have linked RP-induced ROS production with the activation of NADPH oxidase [28,29] and recent reports indicate that CaMKII can activate several isoforms of this oxidase [30,31]. In agreement with this possibility we showed that inhibiting CaMKII with either KN93 or AIP, prevented RP-induced ROS production whereas KN92 failed to prevent this increase. Furthermore, RP-induced ROS production was not observed in AC3-I myocytes compared AC3-C myocytes, where RP significantly enhanced ROS production. Taken together, these results indicate that RP-induced ROS production is CaMKII-dependent. It is now widely accepted that the elevation of ROS is a critical factor in cardiac damage and cell death [32]. Accordingly, ROS scavenging has been shown to attenuate cardiomyocyte apoptosis [26] and contractile dysfunction [33]. Our results show that the ROS scavenger, MPG, prevents both the increase in cell death and the increase in the apoptotic indexes, caspase 3 activity and Bax–Bcl-2 ratio, demonstrating a causal role for enhanced ROS production in RP-induced cell death and apoptosis.

The underlying mechanisms for CaMKII dependent apoptosis are still not completely understood. Recent experimental evidence from our laboratory, in the setting of ischemia and reperfusion injury, suggests that CaMKII may promote apoptosis by altering the coupling between SR Ca^{2+} release and mitochondrial Ca^{2+} uptake, resulting in mitochondrial Ca^{2+} overload and opening of the mitochondrial permeability transition pore (mPTP) [19]. The interplay between SR and mitochondria under different stimuli has been known for many years to be pivotal in triggering apoptotic signals [34,35]. Supporting the critical role played by the SR/mitochondrial interaction in CaMKII-dependent apoptosis, Zhang et al. also concluded that elevated CaMKII results in RyR2 phosphorylation leading to enhanced SR Ca^{2+} leak and mitochondrial Ca^{2+} elevation, and is associated with exacerbated cell death in transgenic mice lacking phospholamban and overexpressing CaMKIIδc, [18]. Further supporting the role of SR-dependent mitochondrial Ca^{2+} overload in CaMKII dependent cell death, Joiner et al. elegantly showed that CaMKII can also directly regulate mitochondrial Ca^{2+} uptake by the uniporter, leading to mitochondrial Ca^{2+} overload and opening of the mPTP [36]. Thus, we hypothesized that RP-induced cell death is at least in part due to CaMKII dependent post-translational modification of the RyR2 resulting in SR Ca^{2+} leak, mitochondrial Ca^{2+} uptake and Ca^{2+} overload. To test the involvement of the RyR2 in RP-induced cell death we used 2 distinct pharmacological compounds known to modulate RyR2 open probability and to suppress arrhythmias, namely, the anesthetic, tetracaine, and the β-adrenoceptor blocker, carvedilol [37,38]. Interestingly both tetracaine and carvedilol prevented RP-induced cell death (Fig. 4B). We obtained similar results using the minimally β-blocking carvedilol analog [38], VK-II-86, excluding the possibility that the observed beneficial effects of carvedilol were related to its β-receptor blocking capacity and not to its action as a RyR2 stabilizer. To gain further insight into the mechanism underlying CaMKII-dependent apoptosis we assessed Ca^{2+} wave frequency as an index of enhanced Ca^{2+} leak from the SR and examined the contribution of mitochondria using Ca^{2+} uptake and permeability transition pore inhibitors, ruthenium red and bongkrekic acid, respectively. In the present study we observed that RP at 5 and 8 Hz significantly increased Ca^{2+} wave frequency and that these events were significantly reduced by the CaMKII inhibitor KN93. In addition we observed that both ruthenium red and bongkrekic acid were able to prevent RP-induced cell death. Although ruthenium red has been shown to be rather nonspecific, taken together, and similar to the conclusion of Zhang et al. [18] our results suggest that RP-induced CaMKII activation leads, at least in part, to an increase in RyR2 open probability and Ca^{2+} leak from the SR, which could result in mitochondrial Ca^{2+} overload and apoptosis.

RyR2 open probability can be modulated, at least in part, by phosphorylation and/or oxidation [39]. Thus, under RP, CaMKII-dependent increase in RyR2 open probability could be due to direct phosphorylation of the CaMKII specific site, Ser 2814, on the RyR2 and/or by increased ROS production and subsequent RyR2 oxidation. To dissect between these possibilities we used myocytes from transgenic mice with the serine 2814 site mutated to alanine and thus, not phosphorylatable by CaMKII. Surprisingly, these myocytes were not protected against RP-induced cell death. However, RP-induced cell death was prevented in these myocytes when stimulation frequency was increased in the presence of the ROS scavenger, MPG. These results indicate that CaMKII-dependent RyR2 phosphorylation is not sufficient to activate the apoptotic cascade. In contrast, the ability of MPG to reduce RP-induced mortality of 2814a myocytes, suggests that RyR2 oxidation plays a central role in the increase in RyR2 open probability leading to Ca^{2+} leak, and cardiomyocyte death under conditions of RP. Consistent with this interpretation is the observation that in the presence of the ROS scavenger MPG, CaMKII activity remains increased but RP-pacing induced cell death is prevented (see Figs. 4 and 5).

Rapid electrical stimulation has been shown to induce early activation of AKT [4]. Similar to the observations of Kuramochi et al. [4] we observed the RP-induced early activation of AKT. In cardiac myocytes, PI3K/AKT cascades have been implicated in survival signaling in response to ischemia and reperfusion, oxidative stress, hypoxia and β-adrenergic stimulation and to protect against apoptosis. In contrast, CaMKII activation has been shown to be deleterious in all these situations [40]. When using 1 μM of the PI3K inhibitor, Wortmannin, we found that inhibiting the PI3K/AKT cascade exacerbated the deleterious effects of RP. These results suggest that RP-induced cell death is inhibited by the simultaneous activation of PI3K/AKT signaling. Consistently, similar findings from our laboratory have shown that another intervention that promotes CaMKII-dependent cardiomyocyte apoptosis, like chronic ouabain treatment, simultaneously activates PI3K/AKT signaling which plays a protective role by reducing the extent of apoptosis [13]. These results suggest that PI3K/AKT signaling may be downstream of CaMKII and could act as a negative feedback loop. Indeed, recent reports indicate that CaMKII activates PI3K/AKT-dependent survival signaling in bone marrow derived microphages under TNF-α treatment [41]. The fact that PI3K/AKT signaling is able to rescue CaMKII-dependent apoptosis induced by different experimental conditions i.e.: RP and chronic ouabain treatment, leads us to speculate that PI3K/AKT signaling may negatively modulate CaMKII activity. Supporting this possibility, in nervous tissue, nitric oxide (NO), a downstream messenger of PI3K/AKT signaling, has been shown to nitrosylate CaMKII and inhibit its activity [42]. Experiments are currently underway in our laboratory to address the potential role of NO as a regulator of CaMKII activity in cardiac tissue.

In summary, and as shown in the schematic representation in Fig. 8, we conclude that RP-induced Ca^{2+} entry activates CaMKII which promotes apoptosis, at least in part, by enhancing ROS production which alters RyR2 function, leading to enhanced diastolic Ca^{2+} release and mitochondrial Ca^{2+} overload. In addition, RP simultaneously activates an antiapoptotic cascade involving PI3K which is however insufficient to completely suppress apoptosis. Finally, our results showing that stabilizing RyR2 can prevent RP-induced cell death suggest the potential use of these agents for the treatment of the adverse remodeling associated with chronic

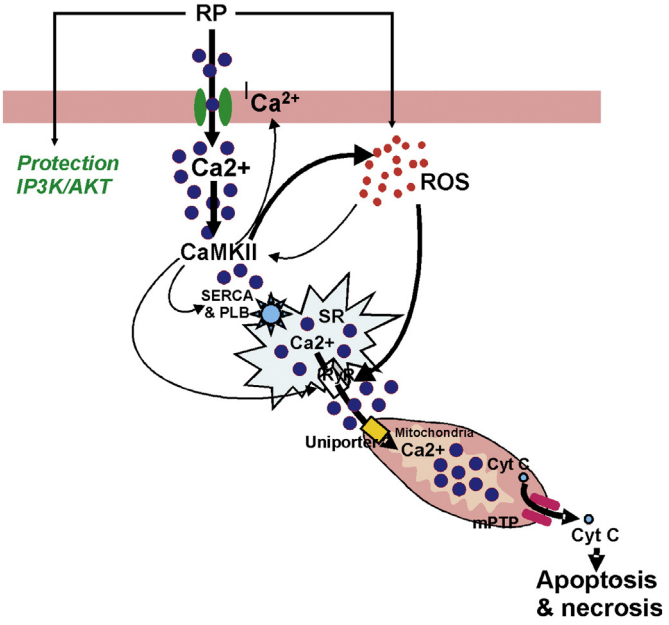

Fig. 8. Flow chart summarizing major sequential events that lead to cell apoptosis/death following rapid pacing. Thick arrows represent preferential routes, showing that rapid pacing enhances Ca^{2+} entry which activates CaMKII which in turn phosphorylates several downstream targets and additionally enhances ROS production. ROS oxidizes the RyR2 and favors SR Ca^{2+} leak which is taken up by the mitochondria resulting in mitochondrial Ca^{2+} overload and activation of the apoptotic cascade. Rapid pacing (RP); phospholamban (PLN); ryanodine receptor (RyR); mitochondrial permeability transition pore (mPTP); cytochrome C (Cyt C).

tachycardia such as that occurring in patients with paroxistic supraventricular tachycardia or atrial fibrillation.

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Disclosure statement

None.

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