Angiotensin II–Induced Oxidative Stress Resets the Ca^{2+} Dependence of Ca^{2+}–Calmodulin Protein Kinase II and Promotes a Death Pathway Conserved Across Different Species

Julieta Palomeque, Omar Velez Rueda, Luciana Sapia, Carlos A. Valverde, Margarita Salas, Martin Vila Petroff, Alicia Mattiazzi

Rationale: Angiotensin (Ang) II–induced apoptosis was reported to be mediated by different signaling molecules. Whether these molecules are either interconnected in a single pathway or constitute different and alternative cascades by which Ang II exerts its apoptotic action, is not known.

Objective: To investigate in cultured myocytes from adult cat and rat, 2 species in which Ang II has opposite inotropic effects, the signaling cascade involved in Ang II–induced apoptosis.

Methods and Results: Ang II (1 μmol/L) reduced cat/rat myocytes viability by ≈40%, in part, because of apoptosis (TUNEL/caspase-3 activity). In both species, apoptosis was associated with reactive oxygen species (ROS) production, Ca^{2+}/calmodulin–dependent protein kinase (CaMK)II, and p38 mitogen-activated protein kinase (p38MAPK) activation and was prevented by the ROS scavenger MPG (2-mercaptopropionylglycine) or the NADPH oxidase inhibitor DPI (diphenyleneiodonium) by CaMKII inhibitors (KN-93 and AIP [autocamtide 2-related inhibitory peptide]) or in transgenic mice expressing a CaMKII inhibitory peptide and by the p38MAPK inhibitor, SB202190. Furthermore, p38MAPK overexpression exacerbated Ang II–induced cell mortality. Moreover, although KN-93 did not affect Ang II–induced ROS production, it prevented p38MAPK activation. Results further show that CaMKII can be activated by Ang II or H_2O_2, even in the presence of the Ca^{2+} chelator BAPTA-AM, in myocytes and in EGTA-Ca^{2+}–free solutions in the presence of the calmodulin inhibitor W-7 in in vitro experiments.

Conclusions: (1) The Ang II–induced apoptotic cascade converges in both species, in a common pathway mediated by ROS-dependent CaMKII activation which results in p38MAPK activation and apoptosis. (2) In the presence of Ang II or ROS, CaMKII may be activated at subdiastolic Ca^{2+} concentrations, suggesting a new mechanism by which ROS reset the Ca^{2+} dependence of CaMKII to extremely low Ca^{2+} levels. (*Circ Res*. 2009;105:1204-1212.)

Key Words: angiotensin II ■ CaMKII ■ apoptosis ■ reactive oxygen species

Experimental evidence indicates that a critical factor in the transition from compensated to noncompensated cardiac hypertrophy is myocyte cell loss by apoptosis.[1] The circulating levels of Ang II are increased in heart failure and may constitute one of the major causes of cell death in this transition.[2] The apoptotic effects of Ang II have been reported to be mediated by different signaling molecules, eg, Ca^{2+}, protein kinase (PK)C-δ, p38 mitogen-activated protein kinase (p38MAPK), or reactive oxygen species (ROS).[2–5] Recent reports have shown that activation of the multifunctional Ca^{2+}/calmodulin–dependent protein kinase (CaMK)II is a common intermediate of diverse death stimuli–induced apoptosis in cardiac cells.[6] Supporting these results, we have demonstrated that CaMKII activation is a critical event in the signaling cascade that leads to apoptosis and necrosis occurring in reperfusion injury.[7] More importantly, we and others have shown that Ang II–induced apoptosis is caused by an increase in CaMKII activity mediated by ROS.[8,9] Recent experimental evidence further indicated that ROS-induced oxidation of methionine residues sustains CaMKII activity in the absence of Ca^{2+}/calmodulin (Ca^{2+}/CaM). However, this action requires previous binding of Ca^{2+}/CaM to expose the autoinhibitory domain of CaMKII for oxidation.[8] Although these experiments suggest the concept that ROS can reset the Ca^{2+} dependence of CaMKII activation, this possibility has never been tested. Moreover, other findings have pointed to the role of phosphatase inhibition in ROS-induced CaMKII activation.[10]

In spite of these important achievements in the understanding of the proapoptotic molecules activated by Ang II, the signaling cascade whereby the peptide produces apoptosis remains uncer-

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From the Centro de Investigaciones Cardiovasculares, Facultad Medicina, Universidad Nacional de La Plata, Centro Científico Tecnológico CONICET La Plata, Argentina.

Correspondence to Dr Martin Vila Petroff, Centro de Investigaciones Cardiovasculares, Facultad de Medicina, La Plata, Argentina. E-mail mvila@aetos.med.unlp.edu.ar

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Non-standard Abbreviations and Acronyms	
AC3-I	autocamtide-3–derived inhibitory peptide
AC3-C	autocamtide-3–derived control peptide
Ang II	angiotensin II
Ca^{2+}_i	intracellular calcium
Ca_iT	intracellular calcium transient
CaM	calmodulin
CaMKII	Ca^{2+}/calmodulin–dependent protein kinase II
DPI	diphenyleneiodonium
IP_3	inositol triphosphate
P	phosphorylated
p38MAPK	p38 mitogen-activated protein kinase
ROS	reactive oxygen species
SB	SB202190

tain. For instance, it is not clear whether the proapoptotic molecules mentioned above are interconnected in one single pathway initiated by Ang II or whether they constitute part of different and alternative cascades by which Ang II exerts its apoptotic action. To examine this issue, we investigated the signaling cascade by which Ang II produces cell death in cultured cardiomyocytes from rat and cat, 2 species in which Ang II produces opposite effects on cardiac contractility.[11,12] The results presented herein, using pharmacological tools and genetic manipulation, show that species with opposite inotropic response to acute Ang II administration share a common apoptotic pathway triggered by the sustained stimulation with the peptide, involving ROS, CaMKII, and p38MAPK. Although these molecules have been previously implicated in the apoptotic effect of Ang II, the present findings demonstrate that they constitute a previously undefined cascade of concatenated events that lead to apoptosis. More importantly, the results indicate that in the presence of ROS, CaMKII may be activated in nominally Ca^{2+}-free conditions. These results reveal a new mechanism by which ROS radically reset the Ca^{2+} dependence of CaMKII to extremely low values.

Methods

Adult cats, Wistar rats, or transgenic mice expressing either a CaMKII inhibitory peptide (AC3-I) or a scrambled control peptide (AC3-C)[8] were used in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No.8-23, revised 1996).

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

ROS- and CaMKII-Dependent Ang II—Induced Apoptosis

Figure 1A shows that 1 μmol/L Ang II significantly reduced the number of viable rat and cat myocytes to 38.9±2.6% and 43.7±3.0%, respectively, after 24 hours of culture. This was prevented in cat myocytes by the Ang II type 1 receptor blocker losartan but not by the AT2 receptor antagonist PD123.319 (41.9±2.9%, $P<0.05$ versus control). Figure 1B and 1C shows that Ang II increased caspase-3 activity and the number of TUNEL-positive nuclei in cat myocytes. Overall, TUNEL-positive nuclei increased from 3.9±0.2% to 10.1±0.1% ($P<0.05$, control versus Ang II), confirming the apoptotic effect of sustained Ang II administration.[2]

Figure 1D depicts typical experiments and overall results showing that Ang II increased ROS production in rat cells. This effect was prevented by 10 μmol/L of the flavoprotein inhibitor diphenyleneiodonium (DPI). Moreover, cotreatment of either rat or cat myocytes with Ang II plus DPI or the free radical scavenger MPG (2-mercaptopropionylglycine) (1 mmol/L) prevented cell death (Figure 1E).

Figure 2A depicts immunofluorescence images from cat cultured myocytes, showing that phosphorylated (P)-CaMKII was increased by Ang II. This increase was prevented by 1 μmol/L of the CaMKII inhibitor KN-93 but not by the inactive analog KN-92. Similarly, KN-93 prevented the increase in P-CaMKII and P-Thr17 of phospholamban, a CaMKII substrate, when evaluated by Western blots (Figure 2B). KN-93, when given alone, failed to significantly affect basal CaMKII activity, as assessed by P-Thr17 (82.2±16.5% of control, n=7). Figure 2C shows that CaMKII inhibition did not prevent the increase in ROS production induced by Ang II in rat myocytes, indicating that Ang II–induced ROS formation is upstream of CaMKII. Figure 2D depicts overall results showing that CaMKII inhibition, either by KN-93 or by a more specific CaMKII inhibitor, AIP (autocamtide 2-related inhibitory peptide) (2.5 μmol/L) also prevented Ang II–induced cell death in both, cat and rat myocytes. Moreover, transgenic myocytes expressing the CaMKII inhibitory peptide (AC3-I) were protected from the deleterious effect of Ang II when compared with myocytes expressing the scrambled control peptide (AC3-C). The images on the right of Figure 2D depict representative experiments showing that CaMKII inhibition prevents the increase in caspase-3 activation and TUNEL-positive cells. Overall, TUNEL-positive nuclei decreased from 10.1±0.1% to 5.73±0.5% and caspase-3 activity from 57.9±17.9% to 27.5±13.9% (Ang II versus Ang II+KN-93).

Taken together, these results indicate that ROS and CaMKII activation are necessary steps of the pathway by which Ang II induces apoptosis, both in cat and rat myocardium.

Ang II–Induced ROS-Dependent Activation of CaMKII Occurs Even in a Nominally Ca^{2+}_i-Free Medium

Results show that prolonged exposure of rat myocytes to Ang II is associated with the activation of CaMKII. However, the source of Ca^{2+} for CaMKII activation is not straightforward in cultured unpaced myocytes. To assess this issue, we evaluated diastolic Ca^{2+} and Ca^{2+}_i transient amplitude (Ca_iT) in electrically field stimulated (0.5 Hz) rat cells, after 24 hours of culture. Ang II did not modify neither diastolic Ca^{2+} nor peak Ca_iT in rat myocytes (diastolic Indo-1 fluorescence, 0.72±0.05 and 0.70±0.05; peak fluorescence, 1.34±0.04 and 1.35±0.05, control versus Ang II). Similarly, sustained administration of Ang II failed to increase diastolic Ca^{2+} or Ca_iT amplitude in cat myocytes, a species in which acute administration of Ang II effectively increases Ca^{2+}_i (Figure 3A and 3B).[11] Furthermore, estimation of sarcoplasmic reticulum Ca^{2+} content by caffeine pulses showed that there was no significant differences between control and Ang II treated cells. In contrast, myocytes stimulated for 24 hours with

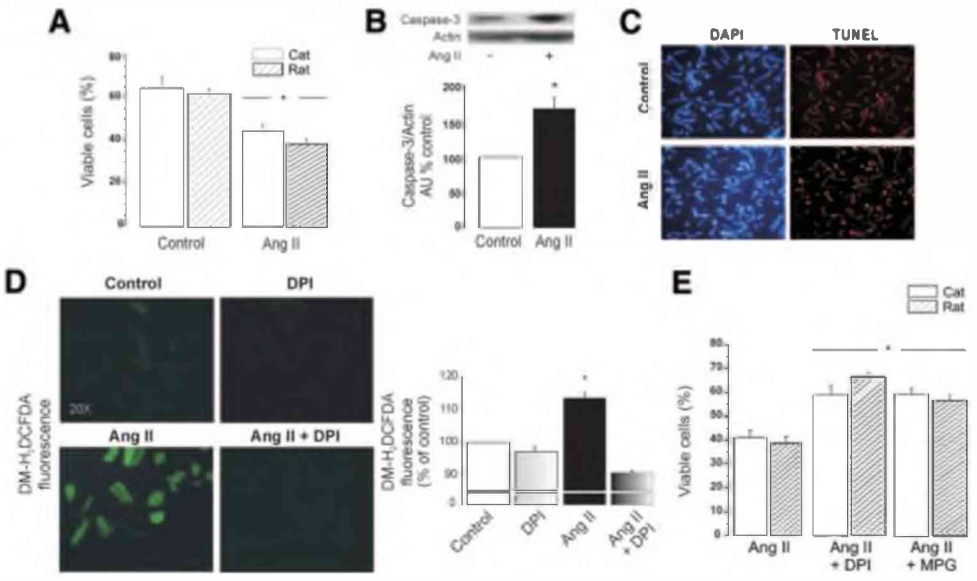

Figure 1. Ang II–induced myocyte apoptosis is dependent on ROS production. A, Cell viability was significantly reduced after 24 hours of culture in the presence of 1 μmol/L Ang II (n=12 and 16 for rat and cat, respectively). B, Representative blots and average data showing that Ang II increased the amount of the 17-kDa cleavage product of caspase-3, indicating caspase-3 activation (n=6). Blots were normalized to actin. C, Typical example of the increase in TUNEL-positive cells produced by Ang II in cat myocytes. D, Representative photographs (left) and average data (right) (n=4), showing the increment in DM-H_2DCFDA fluorescence in myocytes treated with Ang II in the presence and absence of DPI. DPI alone did not significantly affect DM-H_2DCFDA fluorescence. E, Mean results showing that Ang II–induced reduction in rat and cat myocytes viability was significantly prevented by DPI and MPG (2-mercaptopropionylglycine) (n=4 to 6 for each group). In this and the following figures overall data represents means± SEM *P<0.05 vs control.

1 μmol/L isoproterenol (an intervention known to promote Ca^{2+}-dependent apoptosis[13]) showed a significant increase in the amplitude of the Ca_iT and sarcoplasmic reticulum Ca^{2+} content (Figure 3C). Diastolic Ca^{2+} was also measured every 10 minutes during the first hour of incubation with Ang II and every hour in the following 4 hours, to exclude the existence of a possible transitory increase in Ca^{2+}_i at the beginning of the incubation period, undetectable after 24 hours of Ang II stimulation but still responsible for Ang II–induced CaMKII activation. Again, no increase in Ca^{2+}_i was detected in Ang II–treated cells with respect to controls in 4 experiments in which at least 20 cells were observed at each time point (Figure 4A). These experiments indicate that there was no significant increase in Ca^{2+}_i in unpaced myocytes with sustained Ang II stimulation, suggesting that Ang II–induced CaMKII activation could occur in the absence of any increase in Ca^{2+}_i. Because Ang II increases inositol triphosphate (IP_3), it is conceivable that the peptide could produce a compartmentalized increase in Ca^{2+} through the IP_3 receptors, undetectable when measuring bulk cytosolic Ca^{2+}. To explore this possibility, rat myocytes were cultured in the presence and absence of 25 μmol/L 2-APB (2-aminoethoxydiphenyl borate), an IP_3 receptor inhibitor.[14] 2-APB did not prevent Ang II induced cell death: cell viability was 38.9±2.6% (Ang II) versus 36.0±3.2% (2-APB+Ang II) (n=4). These results make unlikely the possibility of a restricted increase in Ca^{2+} produced by IP_3 being the source for CaMKII activation. The effect of Ang II on cell viability and CaMKII activity was also assessed in the presence of 1 μmol/L of the Ca^{2+} chelator BAPTA-AM. BAPTA-AM was added to the culture when the cells were plated. After 1 hour, the medium was changed for a fresh medium containing 1 μmol/L BAPTA-AM and Ang II±KN-93. Control experiments showed that this concentration of BAPTA-AM prevented cell death and the increase in Ca^{2+} levels induced by exposing the cells to the Ca^{2+} ionophore A23128 (Figure 4B). Furthermore, BAPTA abolished twitch contraction and the associated Ca_iT and decreased Indo-1 ratio to values lower than the initial resting levels in electrically stimulated myocytes (results not shown). These experiments indicate that BAPTA-AM was properly loaded into the cell and that Ca^{2+} levels reached subdiastolic values in the presence of BAPTA-AM. Figure 4C shows that Ang II induced a decrease in cell viability and an increase in CaMKII activity and that these effects could be prevented by KN-93. Because Ang II increases ROS (Figure 1D), we assessed whether ROS could promote CaMKII activation and apoptosis in the presence of BAPTA-AM by measuring P-CaMKII and caspase-3 activity by fluorescence microscopy in myocytes stimulated with 200 μmol/L H_2O_2. Both P-CaMKII and caspase-3 activity were increased by H_2O_2 and again these increases were prevented by cotreatment with KN-93 (Figure 4D). These results indicate that Ang II–induced ROS production may activate CaMKII even at subdiastolic Ca^{2+} levels.

To further explore whether the effect of ROS on CaMKII activation was attributable to a direct action of ROS on CaM, we performed experiments in isolated myocytes and in cardiac homogenates in vitro with the CaM inhibitor W-7. Figure 5A and 5B show that in cultured myocytes pretreated with BAPTA-AM, W-7 failed to affect H_2O_2-induced cell death and CaMKII activity (P-CaMKII and P-Thr17), whereas KN-93 prevented both effects. Of note, control experiments showed that when given alone, H_2O_2 produced an increase in CaMKII activity (assessed by P-Thr17 site of phospholamban) of 224±51% (n=3) that was associated with the decrease in cell viability observed in Figure 5A. BAPTA-AM, KN-93, and W-7 failed to significantly affect basal CaMKII activity when given alone. Similar results to those obtained in myocytes were obtained in vitro where H_2O_2 increased P-CaMKII in a nominally Ca^{2+}-free medium (0 Ca^{2+}-EGTA), both in the absence or in the presence of W-7 and no exogenous addition of CaM (Figure 5C). These

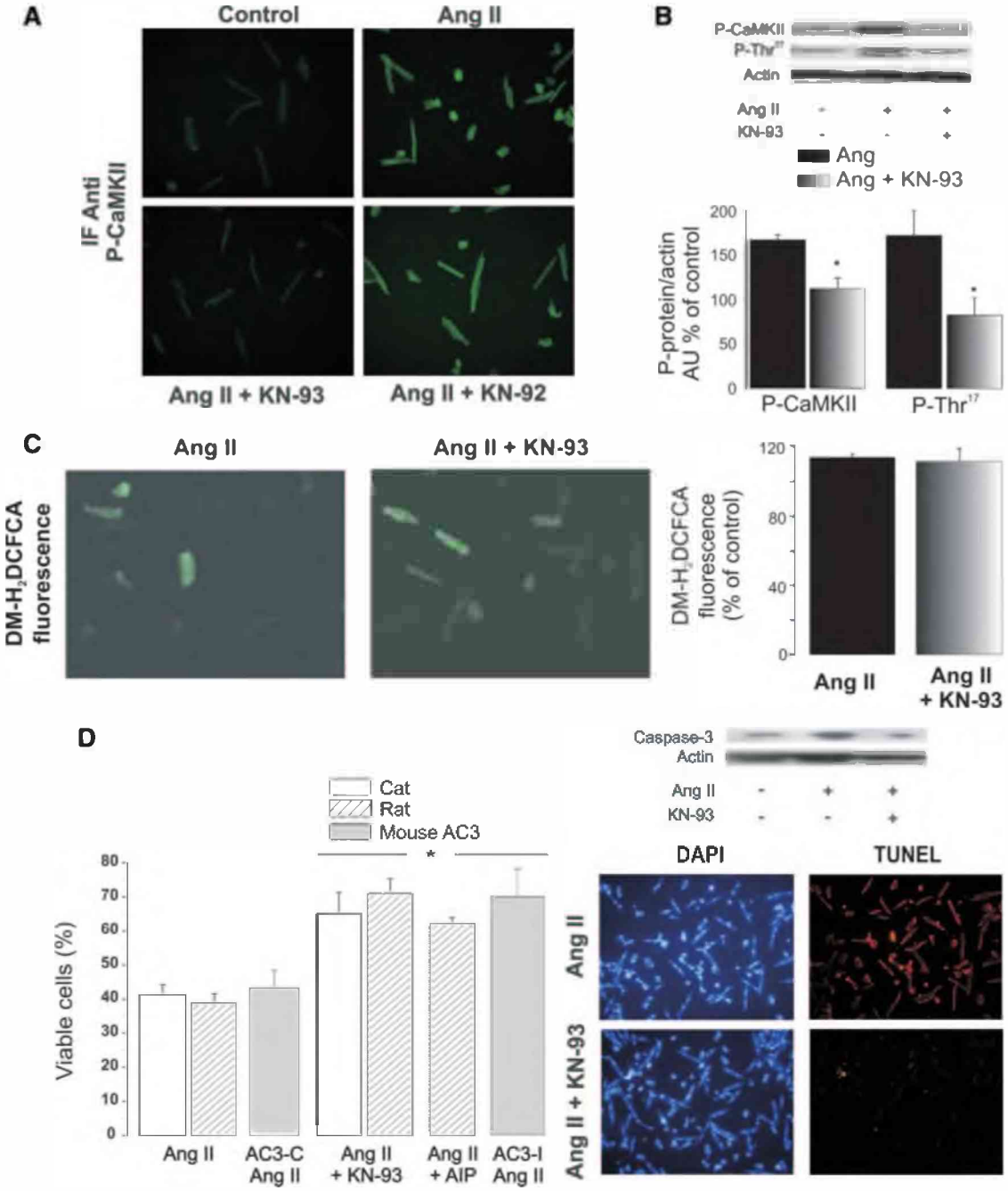

Figure 2. CaMKII activation induced by sustained stimulation with Ang II produces cell death and is dependent on ROS production. A, Immunofluorescence staining with anti P-CaMKII antibody shows increased fluorescence in cat myocytes in the presence of Ang II. The increase in fluorescence was reduced by the concomitant treatment with KN-93. KN-92 did not affect Ang II–induced increase in immunofluorescence. Similar results were obtained in 2 other independent experiments. B, Typical blots and average data showing the increase in CaMKII activity produced by Ang II, assessed by the increase in P-CaMKII and its substrate, P-Thr17 of phospholamban. The increase in both phosphoproteins was prevented by KN-93 (n=9). C, Fluorescent images and average results showing that KN-93 does not affect Ang II–induced DM-H_2DCFCA fluorescence (n=4). D, Average results from cultured cat, rat and transgenic myocytes showing the increase in mortality produced by Ang II in control conditions and its prevention by CaMKII inhibition with KN-93 or AIP (autocamtide 2-related inhibitory peptide) or by using transgenic mice expressing a CaMKII inhibitory peptide (AC3-I) (n=3 to 7 for each group). On the right, typical Western blots showing the effect of Ang II on the activation of caspase-3. Bottom, Fluorescent images showing the increase in the number of TUNEL-positive cells induced by Ang II and its prevention by KN-93. In B and D, blots were normalized to actin. *$P<0.05$ vs Ang II alone.

results were obtained in homogenates from nonbeating hearts perfused with low Ca^{2+}, nifedipine and EDTA, to favor Ca^{2+}, depletion, and in the presence of 1 to 10 mmol/L of EGTA in the in vitro phosphorylation assay. Because CaM is a protein with many methionine residues susceptible to oxidation,[15] we cannot exclude the possibility that the lack of effect of W-7 could be attributable to the fact that oxidized CaM becomes resistant to the inhibitory effects of W-7. However, in in vitro experiments, W-7 was effective in diminishing by 60.8±9.9% the increase in P-CaMKII produced by Ca^{2+} addition in the presence of H_2O_2.

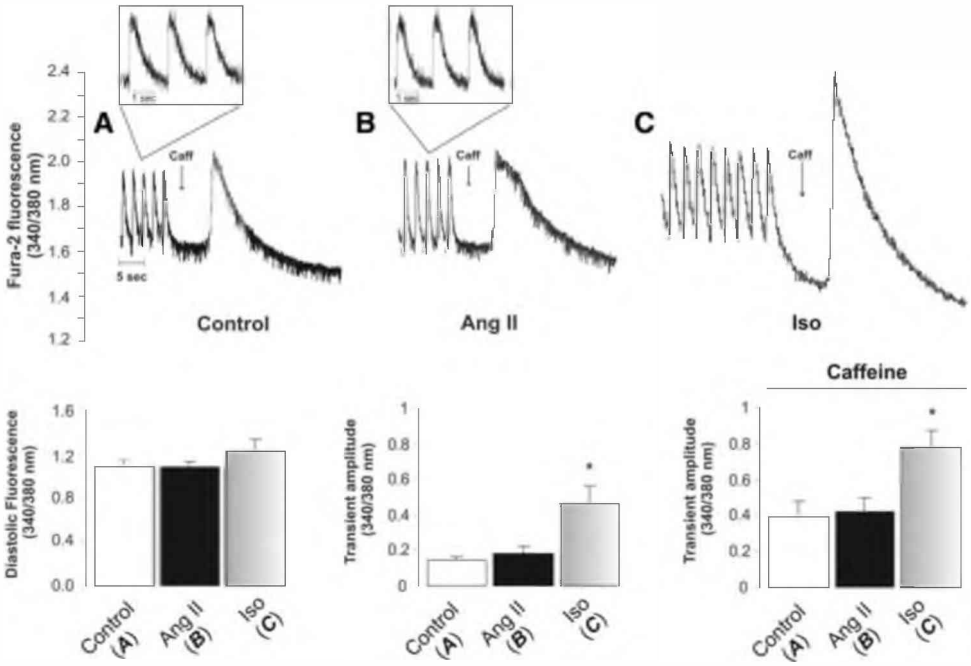

Figure 3. Sustained Ang II stimulation does not modify Ca^{2+} handling. Typical records of Ca_iT and caffeine pulses (Caff) from adult cat cultured myocytes in control conditions (A), treated with Ang II (B), or treated with isoproterenol (Iso) (C) (top). The insets depict individual Ca_iT in an expanded time scale to show that the failure of Ang II to increase Ca_iT amplitude cannot be attributed to incomplete relaxation of the twitches. Bottom, Average values of these experiments. *$P<0.05$ vs the other 2 groups (n=12 cells/per group from 4 different hearts).

These results would support the view that W-7 is able to inhibit CaM, even in the presence of H_2O_2 and would indicate that ROS can either directly activate CaMKII or shift the Ca^{2+}/CaM dependence of CaMKII to extremely low Ca^{2+} and CaM values.

p38MAPK Activation Is Required for Ang II–Induced Cell Death

p38MAPK mediates apoptosis in several models of cardiac stress.[16,17] To evaluate the role of p38MAPK in Ang II–induced apoptosis, 2 different approaches were used: (1) rat and cat myocytes were cultured with Ang II±10 μmol/L of the p38MAPK inhibitor SB202190 (SB) (Figure 6A) and (2) gene transfer was performed to overexpress p38MAPK (Adv.p38) in rat myocytes (Figure 6B). In these experiments, β-galactosidase (Adv.βgal) was overexpressed to serve as control. Figure 6A shows that SB protected cat cardiomyocytes from the deleterious effect of Ang II. Similar results were obtained in rat cells (data not shown). Figure 6B shows that after 24 hours, cells infected with the adenovirus robustly expressed the reporter gene green fluorescent protein (GFP), indicating that the gene of interest was also overexpressed. Results show that although the viability of the p38MAPK-overexpressing cells was not different from that in β-galactosidase–overexpressing cells under control conditions, cells infected with Adv.p38 and incubated with Ang II showed reduced viability compared to β-galactosidase cells. These experiments reveal that p38MAPK is involved in Ang II–induced cell death. Figure 6C further shows that the increase in the activity of p38MAPK induced by Ang II was completely prevented by KN-93. However, SB failed to affect Ang II–induced CaMKII activity (Ang II: 194.1±26.8% of control; Ang II+SB: 180.9±21.5% of control). These findings indicate that activation of p38MAPK is downstream of CaMKII in the Ang II–induced apoptotic signaling cascade.

Discussion

Ang II has been shown to be implicated in many cellular physiological and pathological processes. In the near term, Ang II has been shown to modulate contractility and excitation–contraction coupling. Recent experiments demonstrated that the positive and negative inotropic effects of the peptide occur through different mechanisms. Whereas the positive inotropic effect was associated with an increase in intracellular Ca^{2+},[11,14] negative inotropism occurs in the rat, because of a p38MAPK-mediated decrease in myofilament Ca^{2+} responsiveness.[12] In the long term, Ang II has been associated with cardiomyocyte apoptosis. Since the pioneering studies of Anversa, this apoptotic action was associated with an increase in $Ca^{2+}{}_i$.[2] From then, different molecules have been suggested to mediate the apoptotic effects of Ang II.[4,5,18] However, all of these previous reports failed to explore the sequence of events triggered by angiotensin II type 1 receptor activation that culminate in apoptotic cell death. The present results describe for the first time a sequence of concatenated events by which Ang II produces apoptosis. More importantly, the results indicate that activation of CaMKII may occur at subdiastolic $Ca^{2+}{}_i$ levels. Finally, it is shown that this pathway is common to species in which Ang II has opposite inotropic effects. Thus, the interspecies differences observed in the contractile effect of Ang II, appear to be absent in its apoptotic action, suggesting that a similar apoptotic cascade induced by Ang II may be operative across a wide range of species, including the human.

Ang II–Induced Apoptotic Cell Death Involves ROS-Dependent Activation of CaMKII

The present results demonstrate that Ang II induces ROS production in association with an increase in CaMKII activity. The inhibition of ROS and the prevention of CaMKII activation were both able to inhibit Ang II–induced cell death. Moreover, KN-93 diminished CaMKII phosphorylation and myocyte apoptosis without affecting ROS production, indicating that ROS are upstream of CaMKII (Figures 1 and 2). These results fully confirm the recently discovered ROS-induced CaMKII activation in the apoptotic cascade of Ang II.[8,9,19]

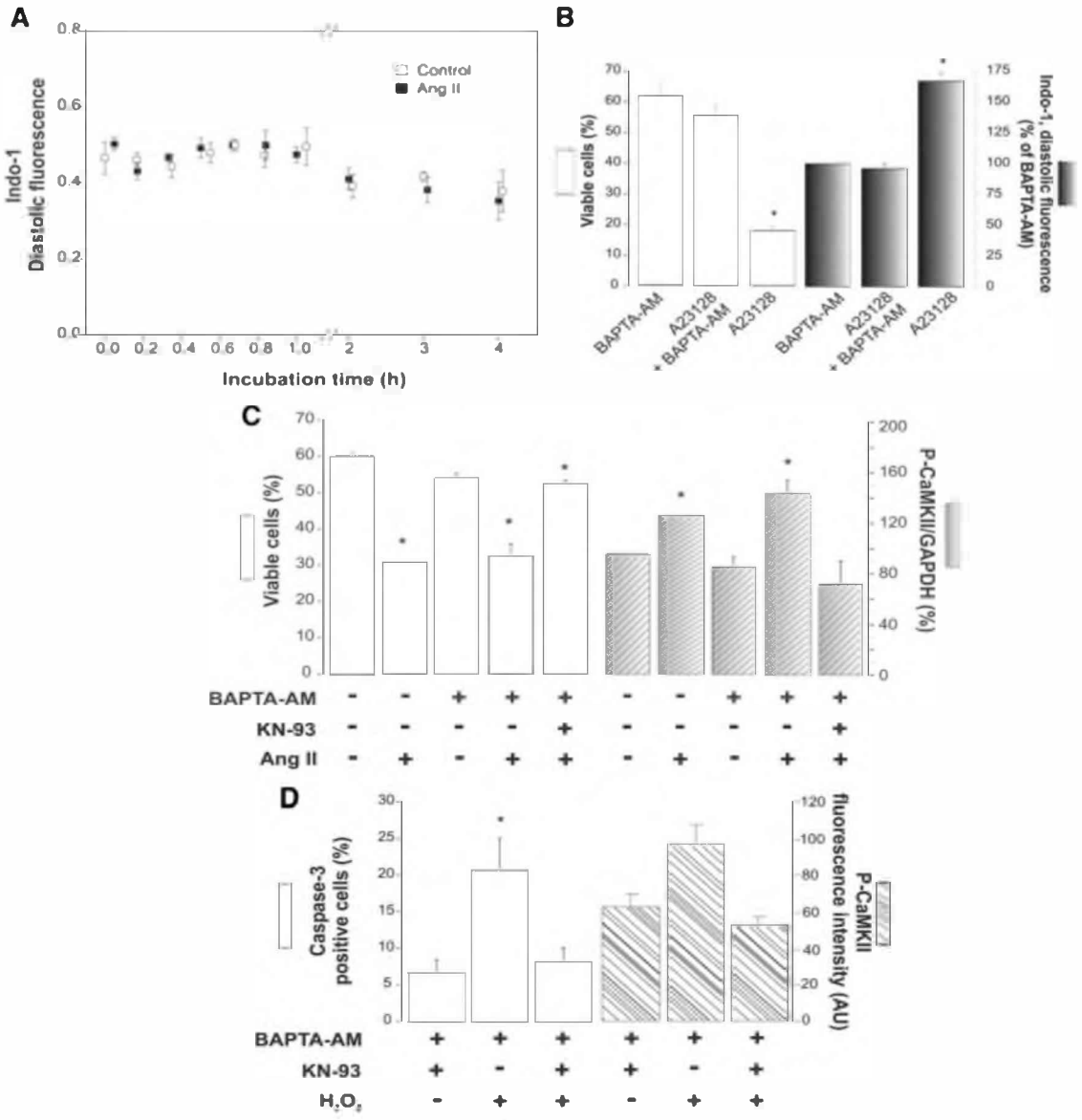

Figure 4. Ang II activation of CaMKII occurs in a nominally Ca^{2+}-free medium. A, Average data from unpaced rat myocytes showing that Ang II did not affect diastolic Ca^{2+} levels during the first 4 hours of treatment. B, Mean results showing cat cell viability and indo-1 diastolic fluorescence in the presence of the Ca^{2+} ionophore A23128±BAPTA-AM. C, Average data of viable cells and P-CaMKII from cat myocytes in the absence and presence of BAPTA-AM, treated with Ang II±KN93. Blots were normalized to GAPDH (n=4 in each group). D, Average results from fluorescence analysis of caspase-3 activity and P-CaMKII in cat cells in the presence of BAPTA-AM and stimulated with H_2O_2±KN-93. P-CaMII fluorescence data were expressed as mean fluorescence intensities. *$P<0.05$ vs the other groups.

CaMKII Activity Can Be Increased by ROS in Nominally Ca^{2+}_i-Free Conditions and After Inhibition of CaM

Recent experiments indicate that sustained activation of CaMKII by ROS occurs by a modification of CaMKII at M281/282 residues. CaMKII activation by ROS appears to require previous "opening" of the kinase by Ca^{2+}/CaM to allow access to the autoinhibitory domain for oxidation.[8] Because our experiments were performed in quiescent myocytes, the source of Ca^{2+} required to initially activate the kinase was not obvious. Indeed, when Ca^{2+}_i was measured in both species after 24 hours of Ang II stimulation, no increases in either diastolic and systolic Ca^{2+} or sarcoplasmic reticulum Ca^{2+} content was detected in stimulated myocytes (Figure 3). These results prompted us to explore in depth the putative source of activating Ca^{2+}, considering different hypotheses. (1) The increase in Ca^{2+} occurred within the first hours during the culture period. This possibility is supported by experiments describing an increase in diastolic Ca^{2+} after incubation with Ang II.[2] (2) There is a compartmentalized increase in Ca^{2+}, not detectable by our epifluorometric measurements of global cytosolic Ca^{2+} but nevertheless able to activate CaMKII. A release of Ca^{2+} by IP_3 receptors is a putative mechanism by which Ang II may produce a restricted increase in Ca^{2+}_i.[20] (3) ROS may alter the affinity of CaM for CaMKII. It is known that CaM weakly associates with the CaM binding domain of the kinase even before binding to Ca^{2+}[21] and several cellular processes are regulated by Ca^{2+}-free CaM.[22] (4) Ang II does not affect basal Ca^{2+} levels but, through the increase in ROS, produces an inhibition of phosphatases that would allow the phosphorylation of CaMKII at resting Ca^{2+}. This possibility

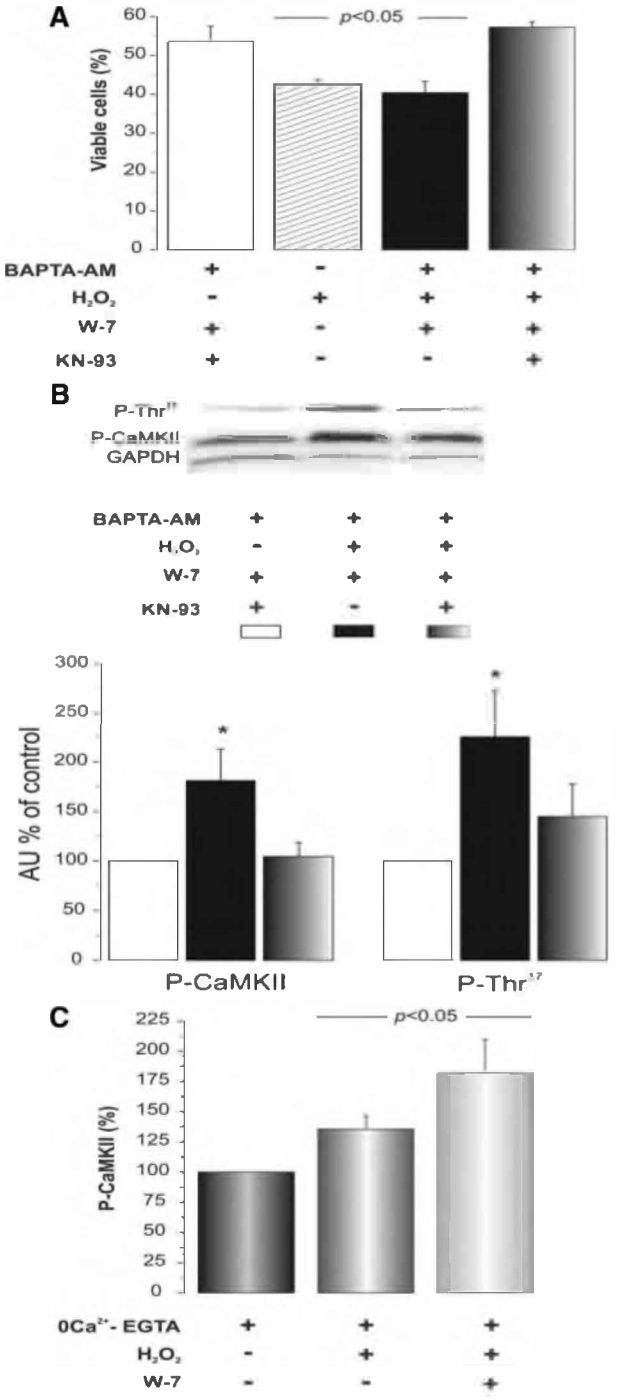

Figure 5. H_2O_2 induces CaMKII activation in the presence of BAPTA-AM and W-7. A, Average data of cat cell viability indicating that H_2O_2 is able to induce cell death mediated by CaMKII activation even in the presence of the Ca^{2+} chelator BAPTA-AM and after CaM inhibition. B, Typical blots and average data from cell cultures showing the increment in the phosphorylation of CaMKII (P-CaMKII) and Thr17 of phospholamban (P-Thr17) because of H_2O_2 stimulation in the presence of BAPTA-AM and W-7. C, Mean data of in vitro CaMKII phosphorylation induced by H_2O_2 in the presence of EGTA and W-7 in cardiac homogenates. *$P<0.05$ vs the other groups.

was suggested in lymphocytes.[10] (5) There might be conditions in which ROS may activate CaMKII directly without the requirement of Ca^{2+}/CaM. (6) ROS may radically reset the CaMKII Ca^{2+} dependence to subphysiological values.

The first 2 hypotheses are not supported by the present results because: (1) we did not detect any significant increase in diastolic Ca^{2+} levels during the first 4 hours of incubation with Ang II and (2) inhibition of IP_3 receptors failed to prevent Ang II–induced cell death. More importantly, incubation of myocytes with BAPTA-AM or of cardiac homogenates with 0 Ca^{2+}-EGTA was unable to inhibit both cell death and CaMKII activation induced by Ang II and H_2O_2, which makes an undetected compartmentalized increase in Ca^{2+} an unlikely candidate. The finding that the inhibition of CaM with W-7 in the presence of BAPTA-AM did not affect H_2O_2-induced CaMKII phosphorylation (Figure 5) would exclude the third possibility, ie, a direct CaM regulation by ROS. Indeed, recent experiments showed that oxidation of CaM diminishes rather than induces CaM-dependent CaMKII activation.[23] The fourth possibility, ie, an increase in CaMKII activity caused by a ROS-induced phosphatase inhibition able to increase the phosphorylated state of the kinase, is hard to reconcile with the fact that autophosphorylation of the kinase requires previous activation.[24] An inhibition of phosphatases would only be able to increase CaMKII activity, measured as P-CaMKII, if the kinase was previously activated to trigger its autophosphorylation (see further discussion below). According to this, one might conclude, at first sight, that Ang II induces a Ca^{2+}/CaM–independent ROS-dependent activation of CaMKII in isolated myocytes. There are at least 3 main reasons, however, that strongly conspire against this possibility. First, experiments by Anderson and colleagues clearly showed that an increase in Ca^{2+}_i is a prerequisite to produce Ang II–induced CaMKII activation.[8] Second, although we used different strategies to decrease Ca^{2+}_i, we are aware of the fact that a complete Ca^{2+}_i depletion is virtually impossible in living systems.[25] Third, we were able to prevent Ang II–dependent CaMKII activation by KN-93, known to inhibit CaMKII by competing with Ca^{2+}/CaM. Thus, our results strongly suggest that the presence of ROS resets the dependence of CaMKII to Ca^{2+} to extremely low, subdiastolic Ca^{2+}_i levels. In this scenario, it is conceivable that an inhibition of phosphatases by ROS could also contribute to sustained CaMKII activation.[10] In line with this idea, recent experiments indicate the need of phosphatase inhibition for the progression of CaMKII autophosphorylation.[26] Of note, buffering Ca^{2+}_i (EGTA or BAPTA-AM) (Figures 4 and 5) may decrease the activity of the Ca^{2+}-dependent phosphatase PP2B, which, by increasing inhibitor-1 phosphorylation, would inhibit PP1, one of the main phosphatases negatively involved in CaMKII phosphorylation.[27] This mechanism may be responsible for part of the basal phosphorylation levels of CaMKII or Thr17 site of phospholamban observed under these conditions. A contribution of phosphatases to the enhanced phosphorylation of these proteins over basal values, under the same Ca^{2+} buffering conditions or in the presence of phosphatase inhibitors (Figure 5C), is more difficult to conceive. However, based on the present results their contribution cannot be completely excluded.

Apoptotic Role of p38MAPK

Our results using SB and Ad.p38 demonstrate that p38MAPK is a crucial step in the apoptotic cascade triggered by sustained Ang II stimulation. Because KN-93 significantly decreased p38MAPK activity, whereas SB has no effect on Ang II–

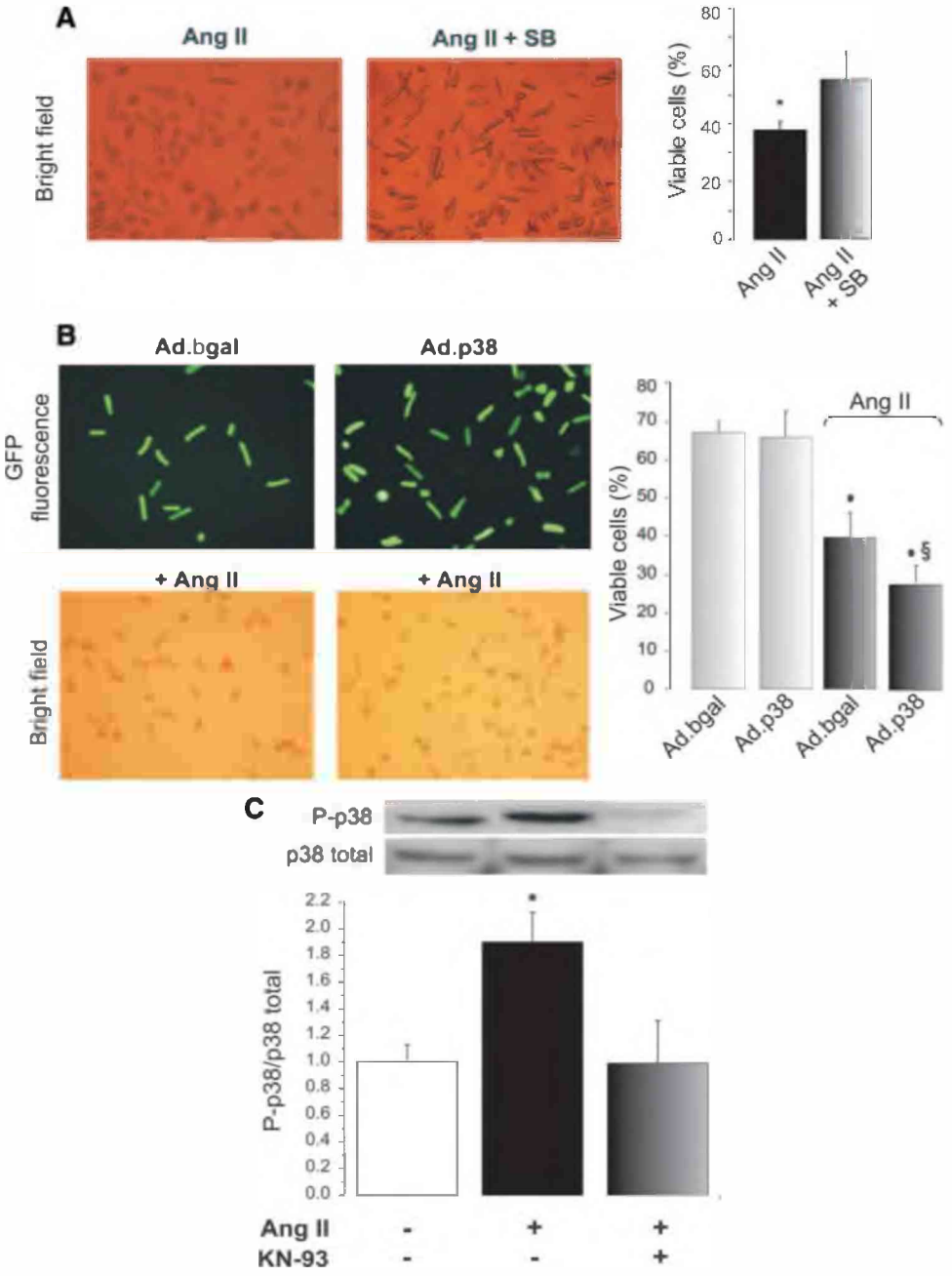

Figure 6. p38MAPK activation is responsible for Ang II–induced cell death and is dependent on CaMKII activation. A, Representative photographs and average data from cat myocytes showing that the Ang II–induced reduction in cell viability can be prevented by the p38MAPK inhibitor SB (n=4 in each group). B, Representative photographs and average results of rat myocytes transfected with Ad.βgal or Ad.p38. Green fluorescent protein (GFP) fluorescence indicates that the transfection efficiency (upper) and bright field images (lower) are representative of the effect of Ang II on cell viability in βgal and p38MAPK-overexpressing cells. Bar graph shows the average results of these experiments (n=3). *$P<0.05$ vs Ad.βgal; §$P<0.05$ vs Ad.p38. C, Typical blots and average data of the effect of Ang II on p38MAPK activity. Effect of Ang II±KN-93 on p38MAPK activation. *$P<0.05$ vs the other groups (n=6).

induced CaMKII activity, p38MAPK would be located downstream CaMKII activation (Figure 6). The potential for CaMKII to activate p38MAPK is not unique to this study. Several reports have shown that p38MAPK can be regulated by CaMKII through the activation of different intermediate molecules, including, apoptosis signal-regulating kinase 1,[28] Janus kinase 2,[29] and protein kinase R.[30] Although the signaling sequence downstream of p38MAPK was not studied in the present experiments, previous reports indicate that CaMKII activates p38MAPK,[29] which, in turn, activates mitochondrial death pathway by producing an unbalance between the expression of pro- and antiapoptotic proteins, Bax and Bcl-2.[31] These results would suggest a downstream location of p38MAPK in the cascade of events initiated by Ang II that leads to apoptosis.

In summary, the present results describe for the first time the sequence of events whereby Ang II mediates apoptosis in cardiac cells. In this cascade, Ang II–induced ROS production appears as the initial trigger for the subsequent activation of CaMKII and the onset of the apoptotic process. The results presented herein further demonstrate that in the presence of ROS, activation of CaMKII may occur without the need of the conventional increase in Ca^{2+} and only requires extremely low Ca^{2+}/CaM levels, revealing a novel and previously unrecognized mode of CaMKII activation in which ROS reset the Ca^{2+} dependence of CaMKII to subphysiological concentrations. Finally, this study shows that the Ang II–induced signaling pathway that results in cardiomyocyte apoptosis is conserved across different species and is operative independently of the inotropic responses elicited by the peptide. It is tempting to speculate that similar apoptotic signaling could occur and even be exacerbated in the failing heart, where Ang II levels, as well as CaMKII and p38MAPK activities, are increased.

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Disclosures

None.

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