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Original Article

Reactive oxygen species partially mediate high dose angiotensin II-induced positive inotropic effect in cat ventricular myocytes

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ABSTRACT

Background: Reactive oxygen species, such as superoxide, are being increasingly recognized as key components of a vast array of signaling pathways. Angiotensin II is a well-recognized stimulus for superoxide production through NADPH oxidase activation and opening of the mitochondrial ATP-sensitive potassium channels (mK_{ATP}). A role for this mechanism has been proposed to explain several physiological effects of the peptide. The aim of this study was to evaluate the involvement of this mechanism in the inotropic response to 100 nmol/L angiotensin II.
Methods: Sarcomere shortening and intracellular pH (BCECF-epifluorescence technique) were evaluated in isolated cat ventricular myocytes placed in a perfusion chamber on the stage of an inverted microscope. Myocardial superoxide production was evaluated by the lucigenin quimioluminiscence method.
Results: Angiotensin II (100 nmol/L) increased~70% sarcomere shortening, effect that was only partially prevented by NADPH oxidase inhibition, mK_{ATP} channel blockade or inhibition of the cardiac Na^+/H^+ exchanger (NHE-1). Moreover, angiotensin II stimulates NHE-1 activity by a NADPH oxidase-dependent mechanism. Myocardial superoxide production was also increased by angiotensin II, and this action was completely prevented either by NADPH oxidase inhibition or mK_{ATP} channel blockade.
Conclusions: The positive inotropic response to 100 nmol/L angiotensin II is due to both ROS/NHE-1 dependent and independent pathways, this being a point of divergence with the signaling previously described to be triggered by lower concentrations of angiotensin II (i.e.: 1 nmol/L).

1. Introduction

Reactive oxygen species (ROS) are produced in all aerobic organisms as a result of incomplete reduction of oxygen during respiration. ROS include free radicals such as superoxide (O_2^-) and non-radical compounds such as hydrogen peroxide. ROS are being increasingly recognized as key regulators of an enormous array of intracellular proteins and signaling pathways, both under physiological and pathological conditions (for a review see [1]). The main sources of myocardial ROS are the mitochondrial electron transport chain, NADPH oxidase (NOX), xanthine oxidase and uncoupled nitric oxide synthase. Angiotensin II (Ang II) is a classical stimulus for NOX and superoxide production [2–4]; and a role for this intracellular signaling pathway has been proposed to explain several physiological effects of the peptide [2,5,6].

Both in vascular smooth muscle and myocardium it has been recently described a phenomenon called "ROS-induced ROS release" that seems to play a key role in intracellular signaling [4,7–9]. It is proposed that ROS generated by the membrane NOX induce the opening of mitochondrial ATP-sensitive potassium channels (mK_{ATP}) which in turn triggers the production and release of greater amounts of ROS from the mitochondria with pathophysiological consequences [4,8,9]. In this regard, there is evidence supporting that some effects of Ang II, including kinases activation, are suppressed not only by NOX inhibition with apocynin but also by the blockade of the mK_{ATP} with 5-hydroxydecanoate (5-HD) or glibenclamide, reinforcing the importance of the ROS-induced ROS release mechanism. On line with this concept it was demonstrated that the ROS-induced ROS release mechanism underlies the increase in contractility and Ca^{2+} transient characteristic of the Anrep effect (slow force response to stretch) [10]. Myocardial stretch through the activation of Ang II AT1 receptors stimulates NOX-dependent mitochondrial ROS release and myocardial Na^+/H^+ exchanger-1 (NHE-1) activity. The NHE-1 is a membrane protein that catalyzes the electroneutral exchange of intracellular protons for extracellular Na^+. NHE-1 hyperactivity leads to intracellular accumulation of Na^+, which increase intracellular Ca^{2+} via the Na^+/Ca^{2+} exchanger (NCX) and consequently a positive inotropic effect is developed [11]. Although the NHE-1 is relatively quiescent under basal conditions, it is activated by the increase in intracellular H^+ concentration as well as by several posttranslational modifications usually due to neurohumoral stimulation (i.e.: Ang II, endothelin, and β-adrenergic stimulation; for a review, see [12]). Moreover, there are several reports supporting a link between ROS and NHE-1 activation

Abbreviations: ROS, reactive oxygen species; NOX, NADPH oxidase; Ang II, angiotensin II; mK_{ATP}, mitochondrial ATP-sensitive K^+ channels; 5-HD, 5-hydroxydecanoate; NHE-1, Na^+/H^+ exchanger-1; NCX, Na^+/Ca^{2+} exchanger; pH_i, intracellular pH.

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[13–16]. It is proposed that ROS induce activation of the MEK-ERK1/2-$p90^{RSK}$ pathway, which increases NHE-1 activity via a phosphorylation process [16,17]. Interestingly, the increase in force that takes place during the Anrep phenomenon may be mimicked by 1 nmol/L exogenous Ang II, a concentration within the range of that found in the interstitial myocardium under physiological conditions [18].

The aim of the present study, which was performed in isolated adult cat ventricular myocytes, was to get insight into the mechanism involved in the positive inotropic effect of Ang II in the concentration that induces the maximal increase in contractility.

2. Materials and methods

All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the experimental protocol was approved by the Animal Welfare Committee of La Plata School of Medicine. Cats (body weight 3–4 kg) were anaesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight) and hearts rapidly excised when plane three of phase III of anesthesia was reached.

Data for each experimental group was obtained from three to six animals.

2.1. Myocytes Isolation

Cat ventricular myocytes were isolated according to the technique described previously [19].

2.2. Sarcomere shortening

To measure sarcomere length myocytes were placed in a perfusion chamber on the stage of an inverted microscope (Nikon) and continuously superfused with a Krebs-HEPES solution containing (mmol/L) NaCl 146.2; HEPES 10; glucosa 11; KCl 4.7; $NaHPO_4$ 0.35; $MgSO_4$ 1; $CaCl_2$ 1.35 (pH 7.4) after continuous bubbling with 99.5% O_2. The myocytes were stimulated via 2-platinum electrodes on either side of the bath at 0.5 Hz. The sarcomere length was recorded with specific software (Ion Wizard). The myocytes were observed using a video camera connected to the microscope. The sarcomere length was measured in a determined region of the myocyte at 30 °C (TC2, Cell micro controls). The software estimated the most frequent sarcomere length in that region using fast Fourier transform analysis (Ion Optix). The average values of the inotropic response were calculated after 10 minutes of incubation with Ang II or no drug. When inhibitors were used, ventricular myocytes were exposed to them from 5 minutes before Ang II stimulus and maintained up to the end of the experiment.

2.3. Intracellular pH (pH_i) measurements

pH_i was measured in single isolated myocytes superfused with the Krebs-HEPES solution (bicarbonate free) with an epi-fluorescence system (Ion Optix, Milton, MA) following the previously described BCECF-epifluorescence technique. [19] Briefly, myocytes were incubated at room temperature for 10 min with 10 μmol/L BCECF-AM followed by 30 min washout. Dye-loaded cells were placed in a chamber on the stage of an inverted microscope (Nikon. TE 2000-U) and continuously superfused with the Krebs-HEPES solution. The myocytes were stimulated via two-platinum electrodes on either side of the bath at 0.5 Hz. Dual excitation (440 and 495 nm) was provided by a 75-watt Xenon arc lamp and transmitted to the myocytes. Emitted fluorescence was collected with a photomultiplier tube equipped with a band-pass filter centered at 535 nm. The 495-to-440 nm fluorescence ratio was digitized at 10 kHz (ION WIZARD fluorescence analysis software). At the end of each experiment, the fluorescence ratio was converted to pH by in vivo calibrations using the high K^+-nigericin method [19]. As described above, the experiments were performed in HCO_3^- free solution. Under these conditions the NHE-1 is the only alkalinizing mechanism active. NHE-1 activity was assessed by evaluating the rate of pH_i recovery from an ammonium pre-pulse-induced acid load [20]. Transient (3 min) exposure of myocytes to 20 mM NH_4Cl was used for this purpose. Proton efflux (J_H) was calculated as $dpH_i/dt \times \beta_i$ and comparison among different groups was done at a common pH_i of 6.8. The dpH_i/dt at each pH_i was obtained from an exponential fit of the recovery phase. βi is the intracellular buffering capacity of the myocytes and was measured by exposing cells to varying concentrations of NH_4Cl in Na^+-free HEPES bathing solution. βi at different levels of pH_i were estimated from the least squares regression lines βi vs. pH_i plots [21]. When used, the inhibitors were added to cells 10 minutes before NH_4Cl exposure and remained present throughout the rest of the protocol.

2.4. Measurement of ROS production

Myocardial O_2^- production was measured by the lucigenin–enhanced chemiluminescence method in cardiac tissue slices from the left ventricle (1 × 5 mm) as previously described [10]. Since high lucigenin concentrations (>20 μmol/L) may favor redox cycling, we used 5 μmol/L lucigenin, at which the amount of artifacts has been proven insignificant [22]. For each intervention evaluated the lucigenin-chemiluminescence signal in arbitrary units was normalized to milligrams of dry weight tissue per minute and

2.5. Chemicals

All drugs used in the present study were analytical reagent. Ang II, lucigenin (5 μmol/L) and 5-HD (100 μmol/L), were purchased from Sigma; apocynin (300 μmol/L; Fluka); losartan (1 μmol/L; Merck); glibenclamide (50 μmol/L); RBI, USA) and cariporide (10 μmol/L HOE642, kindly donated by Aventis Pharma). Either Krebs-HEPES buffer or dimethyl sulfoxyde (DMSO) were used to prepare drug dilutions. The final DMSO concentration, when used, was kept <0.1%.

2.6. Statistics

Results are expressed as mean±SEM. The Student *t* test or 1-way ANOVA followed by the Student-Newman-Keuls test were used when corresponding. Significance level was set at $P<0.05$.

3. Results

The effect of Ang II upon contractility was explored in isolated cat ventricular myocytes. Ang II induced a concentration-dependent increase in sarcomere shortening, reaching the maximal effect at a concentration of 100 nmol/L (Fig. 1). Since the positive inotropic effect

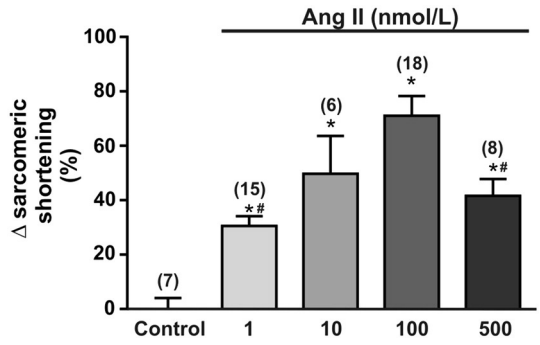

Fig. 1. Inotropic effect of different concentrations of Ang II in isolated cat cardiomyocytes. Note that the maximal effect upon sarcomere shortening was obtained with 100 nmol/L Ang II. Numbers between brackets denote the number of cardiomyocytes per experimental group. * means $P<0.05$ vs. control, # means $P<0.05$ vs. 100 nmol/L Ang; ANOVA.

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induced by 500 nmol/L Ang II was lower than that observed with 100 nmol/L, we decided to perform the rest of the experiments with the latter. An increase in sarcomere shortening of~70 % compared to control cardiomyocytes was detected in the cardiomyocytes exposed to 100 nmol/L of the hormone (Fig. 2A and D). The blockade of the Ang II AT1 receptor with losartan was able to prevent the increase in sarcomere shortening induced by 100 nmol/L angiotensin II (Fig. 2B and D). Moreover, this positive inotropic effect seems to be partially mediated by activation of the NOX since apocynin was able to significantly reduce, although not abolish, it (Fig. 2C and D). As it has been already demonstrated NOX-dependent ROS are able to induce mitochondrial ROS production through a mKATP channel-dependent mechanism, phenomenon known as ROS-induced ROS release [7]. Therefore, the effect of two pharmacologically unrelated mKATP channel blockers (glibenclamide and 5-HD) upon Ang II-induced contractility was explored. Again, both compounds decreased Ang II positive inotropic response by approximately 50%, remaining a similar portion of the contractility effect unchanged (Fig. 3). None of these inhibitors significantly affected contractility in control conditions [111±3% (n=3) and 90±7% (n=5) vs. 100±5% (n=7), for glibenclamide, 5-HD and control respectively]. This is interesting since under the same model it was previously shown that the positive inotropic effect induced by a lower concentration of Ang II (1 nmol/L) can be completely prevented by the ROS scavenger MPG [N-(2-mercapto-propionyl)glycine]. It was also shown that the activation of the NHE-1 played a critical role in the increase in contractility by favoring the reverse mode of the NCX through the rise in intracellular Na^+ concentration. The NCX operating in its reverse mode introduces Ca^{2+} to the cytoplasm that impacts in force development. Therefore we explored the effect of NHE-1 inhibition on the positive inotropic effect of 100 nmol/L Ang II. Cariporide, a specific inhibitor of the NHE-1 did not alter basal contractility (104±6.8% vs. control, n=6); however it did partially reduce Ang II effect similarly to NOX and mK_{ATP} channel inhibitors (Fig. 3). In order to reinforce this result, we then investigated the influence of 100 nmol/L Ang II on NHE-1 activity by measuring pH_i. Steady-state pH_i was slightly but significantly higher in cardiomyocytes exposed to 100 nmol/L Ang II compared to untreated controls (Fig. 4). Under this experimental condition—Krebs-HEPES buffer with nominal absence of bicarbonate in the medium—the only active alkalinizing mechanism is the NHE-1, revealing the increase in steady-state pH_i a stimulatory effect of Ang II upon the exchanger. To further explore this action we analyzed proton efflux during pH_i recovery from an acute acid load in the presence and absence of 100 nmol/L Ang II. Fig. 5A shows representative recordings of pH_i in cardiomyocytes transiently exposed to NH_4Cl to induced intracellular acidosis. Similarly to the inotropic response, Ang II increased NHE-1-dependent proton efflux (J_{H+}) through a NOX-dependent mechanism, since its stimulatory effect was completely prevented by inhibition of NOX with apocynin. Cardiomyocyte intrinsic buffering capacity relative to pH_i was measured to calculate proton efflux through the NHE-1 (Fig. 5B). The average results of J_{H+} are presented in Fig. 5C.

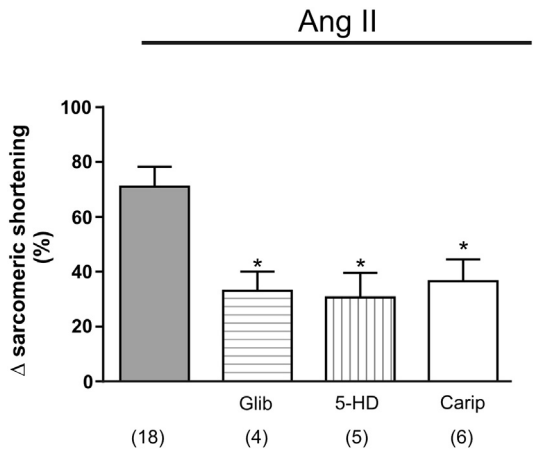

Fig. 3. The signaling pathway evoked by 100 nmol/L Ang II only partially depends on mK_{ATP} channel opening and NHE-1 activity. Both mK_{ATP} with glibenclamide (Glib) or 5-HD and NHE-1 inhibition with cariporide (Carip) significantly reduced the inotropic response to Ang II. However, a significant portion remained (approximately 50 %) supporting that another pathway was also involved. Numbers between brackets denote the number of cardiomyocytes per experimental group. * means $P<0.05$ vs. 100 nmol/L Ang II; ANOVA.

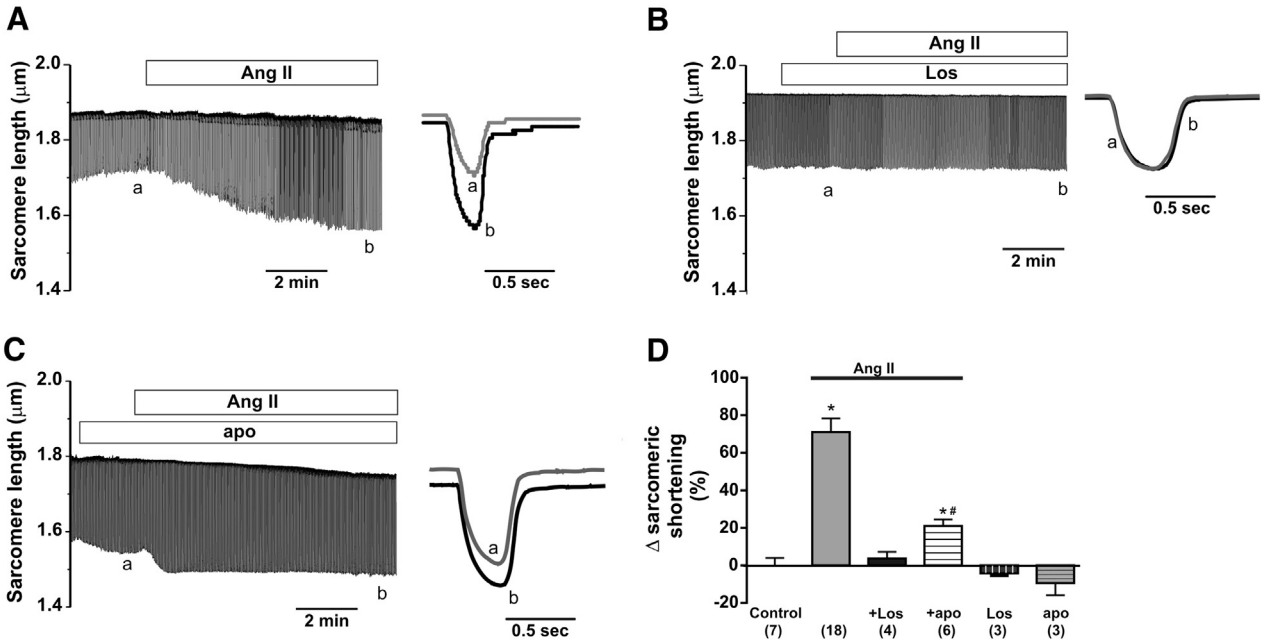

Fig. 2. A. Continuous recordings of myocyte sarcomere shortening before and after the addition of 100 nmol/L Ang II (left). Individual traces corresponding with "a" and "b" are shown in the right (A). Similar recordings were obtained from isolated myocytes preincubated with the AT1 receptor antagonist losartan (Los; B) or the NADPH oxidase inhibitor apocynin (apo; C). D. Average values of the inotropic response to 100 nmol/L Ang II in the presence or absence of losartan or apocynin, compared to control. Neither losartan nor apocynin exert a significant effect on sarcomere shortening in the absence of Ang II. Numbers between brackets denote the number of cardiomyocytes per experimental group. * means $P<0.05$ vs. control, # means $P<0.05$ vs. 100 nmol/L Ang; ANOVA.

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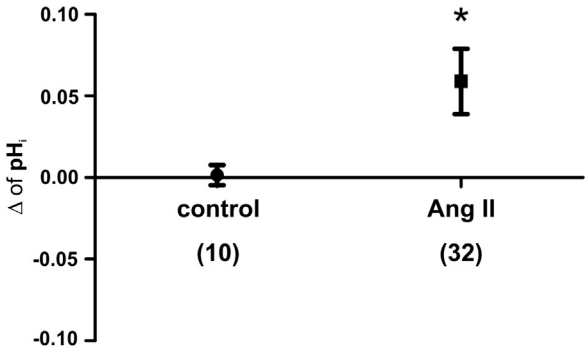

Fig. 4. Ang II (100 nmol/L) induced a slight but significant alkalization of isolated cardiomyocytes. Since intracellular pH (pH_i) recordings were performed in nominally absence of bicarbonate in the medium (Krebs-Hepes buffer) the rise in pH_i should be attributed to an enhanced activity of the NHE-1 in the presence of Ang II. Numbers between brackets denote the number of cardiomyocytes per experimental group. * means $P<0.05$; t-test.

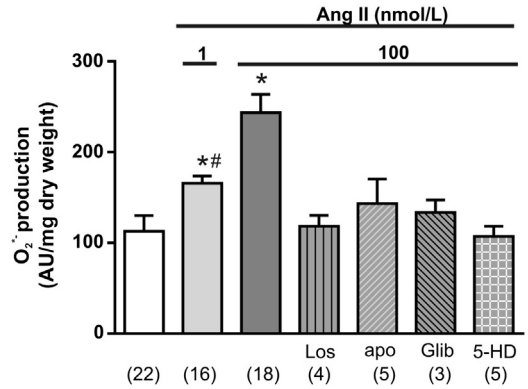

Fig. 6. Ang II dose-dependently increased myocardial superoxide ($O_2^{\cdot-}$) production. The effect of 100 nmol/L Ang II was further explored detecting that it was completely cancelled by AT1 receptor blockade with losartan (Los), NOX inhibition with apocynin (apo) and mK_{ATP} channel blockade with either glibenclamide (Glib) or 5-HD. Numbers between brackets denote the number of experiments per group. * means $P<0.05$ vs. control, # means $P<0.05$ vs. 100 nmol/L Ang; ANOVA.

Finally, we explore the effect of 100 nmol/L Ang II on cat myocardial ROS production. Fig. 6 shows that 100 nmol/L Ang II increased ROS production, effect that was completely canceled not only by the specific blockade of Ang II AT1 receptor with losartan but also by NOX inhibition with apocynin or mK_{ATP} blockade with glibenclamide or 5-HD. In order to explore the existence of a dose dependent response to Ang II on ROS production, similar to what is observed for sarcomere shortening, 1 nmol/L Ang II effect was also evaluated. This concentration of the peptide increased ROS production although to a significantly lower extent than 100 nmol/L Ang II (Fig. 6).

4. Discussion

The present study provides insight into the mechanism underlying the cardiomyocyte inotropic effect of Ang II in a concentration that it is not only the most widely used in the experimental arena but also the one that induces the greater contractility effect in isolated cat cardiomyocytes. Physiological plasma and myocardial tissue concentrations of Ang II have been reported to be in the pmol/L to nmol/L range; however it has been well demonstrated that during pathological conditions these concentrations significantly increase, reaching values even about a hundred of times higher [23]. We detected that 100 nmol/L Ang II, through activation of the AT1 receptors, induces NOX and mitochondrial-dependent ROS production responsible for approximately half of the increase in sarcomere shortening. Moreover, this concentration of the hormone also stimulates the NHE-1 probably through the same pathway, since the increase in NHE-1 activity was prevented by the NOX inhibitor apocynin.

The results presented herein constitute a relevant difference with the mechanism involved in the inotropic response to 1 nmol/L Ang II—a concentration in the range of that in the interstitial myocardium under physiological conditions [18]—that it has been previously described in detail elsewhere [6]. Under those circumstances both ROS

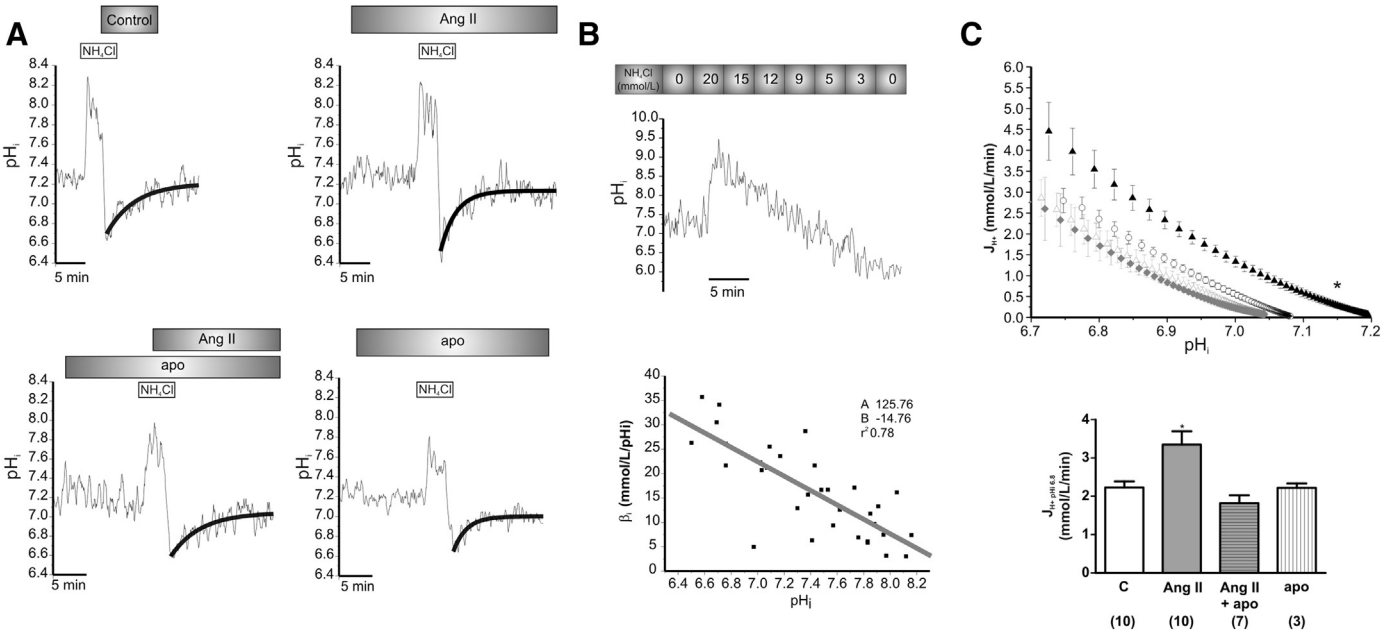

Fig. 5. Ang II stimulated NHE-1 activity during pH_i recovery from intracellular acidosis by a NOX-dependent pathway. Experiments were performed in isolated cat ventricular myocytes in bicarbonate free medium in order to exclude the involvement of any other alkalinizing mechanism. A. Representative recordings for each experimental condition. B. Intrinsic buffering capacity (β_i) relative to pH_i was measured by exposing cardiomyocytes to varying concentrations of NH_4Cl in Na^+-free HEPES pH 7.40 bathing solution (n=32 measurements from 8 isolated cardiomyocytes) as explained in Methods. C. NHE-1 activity measured as proton efflux (J_H^+). Comparison among the different groups was done at a common intracellular pH of 6.8, as explained in Methods. Numbers between brackets denote the number of cardiomyocytes per experimental group. * means $P<0.05$ vs. control: ANOVA.

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and NHE-1 appear to be strictly required to increase contractility. Furthermore, it has been already demonstrated that myocardial stretch shares the intracellular pathway evoke by 1 nmol/L exogenous Ang II to increase contractility [24]. Briefly, myocardial stretch induces AT1 receptor activation that through a chain of events leads to increased production of NOX-dependent mitochondrial ROS, activation of redox-sensitive kinases and stimulation of the NHE-1 which finally increases Na^+ and consequently Ca^{2+} transient amplitude through the NCX operating in reverse mode.

In the case of higher Ang II concentrations (i.e.: 100 nmol/L) it seems that at least two different pathways are activated leading to the inotropic response: one dependent on NOX/mitochondrial ROS production and NHE-1 activation (described above) and the other one being ROS/NHE-1 independent. One possible mechanism involved in the latter could be an increase in L-type calcium current induced by PKC activation through 100 nmol/L Ang II [25]. Nevertheless, further research will be necessary to elucidate the intracellular events and final effectors involved in the latter.

Among NOX family members, NOX2 and NOX4 are the isoforms primarily expressed in cardiomyocytes (for review see Ref. [26]). While NOX2 is mainly localized in the cellular membrane and is sensitive to inhibition by apocynin, NOX4 has been recently described as primarily expressed in cardiac mitochondria; and it is not regulated either by apocynin or post-translational modifications [27]. The immediate product of NOX enzymes is O_2^-, but because of both spontaneous and enzymatic dismutation H_2O_2 is also rapidly generated from this short-lived, unstable compound. We did not characterize the NOX isoform involved in the pathway leading to ROS formation; however, previous available evidence [28,29] and the fact that apocynin suppressed ROS production in our study suggest that NOX2 is the isoform involved. With respect to the ROS-induced ROS released mechanism, although there is no conclusive data supporting that sarcolemmal NOX-derived ROS interacts with the mitochondria, Zhang *et al.* [30] using reconstituted mK_{ATP} channels of bovine heart demonstrated that O_2^- directly stimulates the opening of these channels. The opening of mK_{ATP} channels would allow K^+ entry, mitochondrial swelling and increased ROS production/release [9,31,32].

5. Conclusions

- Ang II exerts its maximal positive inotropic effect at the concentration 100 nmol/L in isolated cat ventricular myocytes.
- Different pathways underlie the increase in contractility induced by Ang II depending on the concentration of the hormone.
- The positive inotropic response to 100 nmol/L Ang II is due to both ROS/NHE-1 dependent and independent mechanisms, this being a point of divergence with the signaling triggered by lower concentrations of Ang II (i.e.: 1 nmol/L).

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