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Original article

Angiotensin II inhibits the electrogenic Na^+/HCO_3^- cotransport of cat cardiac myocytes

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ABSTRACT

The Na^+/HCO_3^- cotransporter (NBC) plays an important role in intracellular pH (pH_i) regulation in the heart. In the myocardium co-exist the electrogenic (eNBC) and electroneutral (nNBC) isoforms of NBC. We have recently reported that angiotensin II (Ang II) stimulated total NBC activity during the recovery from intracellular acidosis through a reactive oxygen species (ROS) and ERK-dependent pathway. In the present work we focus our attention on eNBC. In order to study the activity of the eNBC in isolation, we induced a membrane potential depolarization by increasing extracellular K^+ $[K^+]_o$ from 4.5 to 45 mM (K^+ pulse). This experimental protocol enhanced eNBC driving force leading to intracellular alkalization (0.19 ± 0.008, $n = 6$; data expressed as an increase of pH_i units after 14 min of applying the K^+ pulse). This alkalization was completely abrogated by the NBC blocker S0859 (-0.004 ± 0.016*, $n = 5$; * indicates $p < 0.05$ vs control) but not by the Na^+/H^+ exchanger blocker HOE642 (0.185 ± 0.04, $n = 4$), indicating that we are exclusively measuring eNBC. The K^+ pulse induced alkalization was canceled by 100 nM Ang II (-0.008 ± 0.018*; $n = 5$). This inhibitory effect was prevented when the myocytes were incubated with losartan (AT_1 receptor blocker, 0.18 ± 0.02; $n = 4$) or SB202190 (p38 MAP kinase inhibitor, 0.25 ± 0.06; $n = 5$). Neither chelerythrine (PKC inhibitor, -0.06 ± 0.04*; $n = 4$), nor U0126 (ERK inhibitor, -0.07 ± 0.04*; $n = 4$) nor MPG (ROS scavenger, -0.02 ± 0.05*; $n = 8$) affected the Ang II-induced inhibition of eNBC. The inhibitory action of Ang II on eNBC was corroborated with perforated patch-clamp experiments, since no impact of the current produced by eNBC on action potential repolarization was observed in the presence of Ang II. In conclusion, we propose that Ang II, binding to AT_1 receptors, exerts an inhibitory effect on eNBC activity in a p38 kinase-dependent manner.

1. Introduction

The major transporters responsible for acid extrusion from the cardiac myocytes are the Na^+–H^+ antiporter (NHE) and the Na^+–HCO_3^- cotransporter (NBC), which transport H^+ out and HCO_3^- in the cell, respectively [1,2]. In the absence of HCO_3^-, the only active acid extruder mechanism is the widely studied NHE. However, in a bicarbonate medium, such as blood, both mechanisms, the NHE and the NBC, are equally operative at a pH_i close to basal [3–7].

Several functional studies have suggested that the NBC can either mediate an electroneutral (1 Na^+:1 HCO_3^-) or an electrogenic (1 Na^+:2 HCO_3^-) co-influx of sodium and bicarbonate into cardiac myocytes [2,3,6,8–11]. The presence of at least three isoforms of NBC coexisting in the heart is now accepted; one electroneutral (NBC3 or NBCn1) and two electrogenic (NBC1 or NBCe1 and NBC4 or NBCe2) [5,12]. In our laboratory, we have determined the influence of the electrogenic NBC (eNBC) in the configuration of the rat and cat ventricular action potential (AP), generating an anionic repolarizing current which produces an AP shortening of approximately 25% [9,11]. However, the electrogenic isoform involved in this effect has not been determined yet.

Angiotensin II (Ang II), an octapeptide which is part of the renin-angiotensin-aldosterone system, is an important regulator of cardiac physiology. The effects and intracellular pathways of Ang II involved in the regulation of the cardiac NHE have been well established [13–15]. However, the effects of this hormone on NBC have been less studied. In neonatal rat ventricular myocytes, Ang II was shown to stimulate the NBC through a phosphoinositide-independent pathway after activation of AT_2 receptors [16]. On the other hand, it has been reported that Ang II stimulates Na^+/HCO_3^- co-transport activity in renal [17] and in adult rat ventricular myocytes [18], involving MAPK-dependent pathways. Moreover, we have recently demonstrated that Ang II enhanced pH_i recovery after acidification in adult cat myocytes through a ROS-dependent mechanism [19]. Nevertheless, the cardiac NBC isoform involved in the stimulatory effect of Ang II has not been determined yet. Thus, the objective of the present study was to examine the effect of Ang II on eNBC activity. Although, based in our previous data, we initially hypothesized that Ang II would stimulate the activity of eNBC, we found the opposite effect, since inhibition, rather than stimulation of eNBC by

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Ang II was detected in the present work. Therefore, we propose that Ang II exert opposite effects on cardiac NBC isoforms, stimulation of the electroneutral NBC (nNBC) and inhibition of eNBC.

2. Methods

The investigation conforms 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).

2.1. Cell isolation

Cat ventricular myocytes were isolated according to the technique previously described [20].

2.2. pH_i measurements

pH_i was measured in single myocytes with an epi-fluorescence system (Ion Optix, Milton, MA) using the previously described BCECF technique [21]. Briefly, myocytes were incubated at room temperature for 30 min with 10 μM 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 a solution containing (mM) 5 KCl, 118 NaCl, 1.2 $MgSO_4$, 0.8 $MgCl_2$, 1.35 $CaCl_2$, 10 glucose, 20 $NaHCO_3$, pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2. 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 [22].

The experiments were performed at 30 °C. Bath temperature was set at 30 °C with a Temperature Controller (Cell MicroControls, Norfolk, VA, USA). The cells were paced at 0.5 Hz under field stimulation.

2.3. Ammonium pulse

As described above, the experiments were performed in HCO_3^- buffered solution. Under these conditions, both pH_i regulatory systems are operative, NHE and NBC. In order to examine the NBC activity in isolation all the experiments were performed in the presence of 10 μM HOE642. In parallel experiments performed in HEPES-buffered solution (without HCO_3^-) we corroborated that this concentration of HOE642 completely blocked the pH_i recovery mediated by the NHE (Fig. 1 of the Supplementary material), as supported by previous results from other groups [18,23,24]. The total NBC activity was assessed by evaluating the pH_i recovery from an ammonium pre-pulse-induced acute acid load. Transient (3 min) exposure of myocytes to 20 mM NH_4Cl was used for this purpose. The dpH_i/dt at each pH_i, obtained from an exponential fit of the recovery phase, was analyzed to calculate the net H^+ efflux (J_H), then $J_H = \beta_{tot}\, dpH_i/dt$, where β_{tot} is the total intracellular buffering capacity. β_{tot} was calculated by the sum of the intracellular buffering due to CO_2 (β_{CO2}) plus the intrinsic buffering capacity (βi). β_{CO2} was calculated as, $\beta_{CO2} = 2.3[HCO_3^-]_i$, where $[HCO_3^-]_i = [HCO_3^-]_o\, 10^{pH_i - pH_o}$ [25,26]. βi of the myocytes was measured by exposing the cells to varying concentrations of NH_4Cl in Na^+-free HEPES bathing solution. pH_i was allowed to stabilize in Na^+-free solution before application of NH_4Cl. βi was calculated from the equation $\beta i = \Delta[NH_4^+]i / \Delta pH_i$ and referred to the mid-point values of the measured changes in pH_i. βi at different levels of pH_i were estimated from the least squares regression lines βi vs. pH_i plots (as shown in Fig. 2 of the Supplementary material).

2.4. Potassium pulse

To investigate the eNBC activity in isolation we performed a potassium pulse. Increasing isotonically extracellular K^+ $[K^+]_o$ from 5 to 45 mM produced a depolarization of approximately 60 mV that enhanced the eNBC activity and in turn elevated pH_i. The high K^+ was applied for 14 min and during this period the pH_i was recorded. The data were expressed as an increase of pH_i units in comparison to the zero time point in high K^+ solution. The HCO_3^--buffered solution used in the K^+-induced depolarization experiments contained (mM): 118 NaCl, 5 KCl, 1 $MgSO_4$, 0.35 NaH_2PO_4, 10 glucose, 40 choline chloride, and 20 $NaHCO_3$, pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2. K^+-induced depolarization was assessed by replacing 40 mM choline chloride with 40 mM KCl, maintaining ionic strength.

2.5. Patch-clamp recordings

The Nystatin perforated whole-cell configuration of the patch clamp technique was used for current-clamp recordings with a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, Calif.). Patch pipettes were pulled with a PP-83 puller (Narishige, Tokyo, Japan) and fire-polished with a MF-83 Microforge (Narishige) to a final resistance of 0.5–1 MΩ when filled with a control pipette solution. Membrane voltage (sampling rate = 1 kHz; low pass filter = 1 kHz) were digitally recorded directly to hard disk via an analog-to-digital convertor (Digidata 1200, Axon Instruments) interfaced with an IBM clone computer running pClamp software (Axon Instruments). A pacing rate of 0.2 Hz was applied. Data analysis was performed with pClamp (Clampfit). An Ag/AgCl wire directly in contact with the extracellular solution was used as reference electrode. Since the pipette potential was nulled in external solution, all current-clamp tracings required corrections for junction potential. This was accomplished by filling 20 pipettes with standard internal solution. They were then nulled in internal solution, and the difference in potential on immersion in external solution was recorded. The measured junction potential value was consistently −10 mV, and this value was used to correct all current-clamp data and voltage-clamp protocols. There were no significant differences in the value of junction potential among all the external solutions used in the present work. The HCO_3^--free external solution (HEPES-buffered) contained (mM): NaCl 133, KCl 5, $MgSO_4$ 1.2, $MgCl_2$ 0.8, glucose 10, $CaCl_2$ 1.35, and HEPES 10, pH 7.35 with 5 mM NaOH. The HCO_3^--buffered solution contained (mM): NaCl 118, KCl 5, $MgSO_4$ 1.2, $MgCl_2$ 0.8, glucose 10, $CaCl_2$ 1.35, choline–Cl 15, and $NaHCO_3$ 20, pH was 7.4 after gassing with 95% O_2–5% CO_2. The Nystatin pipette solution contained in mM: KGluconate 130, KCl 10, NaCl 5, $MgCl_2$ 0.5, EGTA 1, HEPES 10, and Nystatin 0.3 mg/ml. The pH was titrated to 7.2 with KOH.

2.6. Statistics

Data were expressed as means ± S.E.M. and were compared with one-way ANOVA followed by Student–Newman–Keuls post-hoc test for unpaired data, except for the patch-clamp data that were compared with Student's t test for unpaired data. A value of $p < 0.05$ was considered statistically significant (two-tailed test).

3. Results

3.1. Isolation of the eNBC activity with the potassium pulse

Fig. 1(A) shows representative traces of continuous pH_i recording of a myocyte exposed to a high extracellular K^+ solution (45 mM). The hyperkalemic-induced depolarization of membrane potential resulted in an alkalization that was reversed upon washout of the high K^+ solution. Fig. 1(B) shows average results of this voltage-dependent

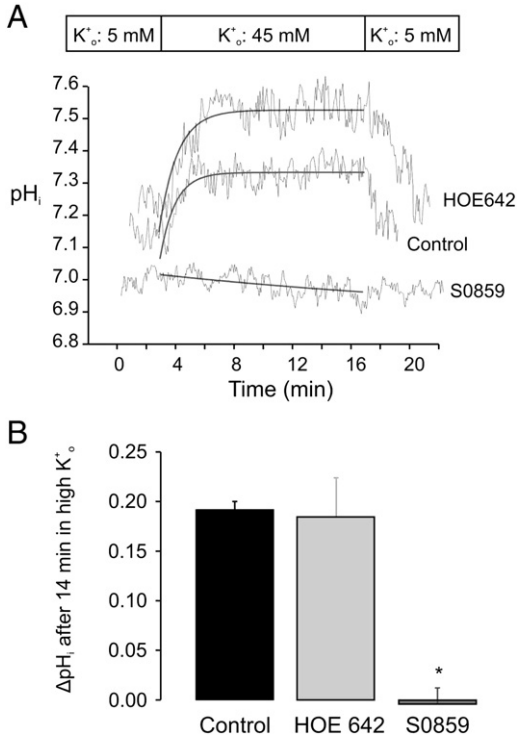

Fig. 1. Isolation of eNBC activity with the potassium pulse. (Panel A) Representative traces of pH_i recorded from myocytes exposed to the potassium pulse (see methods) in control and in the presence of the NHE blocker HOE642 (10 μM) or the NBC blocker S0859 (10 μM). (Panel B) Average data of pH_i alkalization induced by the hyperkalemic-induced depolarization of membrane potential in control (n = 6) and in the presence of the inhibitor of NHE, HOE642 (10 μM, n = 4) or the inhibitor of NBC, S0859 (10 μM, n = 5). Data are expressed as an increase of pH_i units after 14 min in high K^+ solution. * indicates $p < 0.05$ vs. control and HOE642. HOE642 or S0859 were added to the bath solution 10 min before the potassium pulse.

alkalization. As observed in Figs. 1(A) and (B) this increase in pH_i was canceled by pretreatment of the cells with the recently reported NBC blocker S0859 [27], but not by the NHE inhibitor HOE642, indicating that this effect is mediated by selective activation of eNBC.

3.2. Effect of Ang II on eNBC activity

Ang II has been shown to activate NBC in cultured neonatal and adult rat ventricular myocytes [16,18]. Moreover, we have recently demonstrated that total NBC-mediated acid extrusion is stimulated by Ang II in adult cat myocytes [19]. However, no data are available concerning the effect of Ang II on the eNBC. Fig. 2 illustrates the time course of pH_i change under control conditions and after exposing the myocytes to 100 nM Ang II. Surprisingly, Ang II cancelled the pH_i alkalization. This negative effect was inhibited by the blocker of the AT_1 receptors, losartan (1 μM) but not by the AT_2 antagonist, PD123,319 (1 μM), indicating that the hormone, binding to its AT_1 receptors, had an inhibitory effect on eNBC activity. The inset of Fig. 2 shows that neither losartan nor PD123,319 affected the basal rise of pH_i induced by the potassium pulse.

In order to investigate the potential mechanisms underlying the inhibition of eNBC activity, and because we have previously reported the participation of ROS as second messenger molecules in the Ang II-induced enhancement of total NBC activity, we examined the effect of adding the ROS scavenger MPG (2 mM) to the extracellular solution. As shown in Fig. 3, in the presence of MPG, Ang II still exhibited an inhibitory effect on eNBC activity, indicating that the eNBC inhibition is ROS-independent. We also examined the involvement of protein kinase C (PKC) in Ang II-induced inhibition of eNBC. Pre-incubation of the cells with the PKC blocker chelerythrine (2 μM) failed to affect the inhibitory effect of Ang II on eNBC activity (Fig. 3), indicating that the participation of protein Gqα is not involved in this effect. Neither MPG nor chelerythrine affected the basal rise of pH_i induced by the potassium pulse (Fig. 3, inset).

3.3. Role of p38 and ERK1/2 MAP kinases in the Ang II-mediated inhibition of eNBC

Ang II is known to induce the phosphorylation and activation of p38 and ERK1/2 kinases [28]. It has been reported that Ang II activates NBC after stimulation of the ERK pathway in both rat [18] and cat myocytes [19]. In order to determine if these kinases were implicated in the Ang II-induced inhibition of eNBC, we examined the effect of the treatment of the myocytes with U0126 (10 μM) or SB202190 (10 μM), inhibitors of the ERK and p38 signaling cascade, respectively. As illustrated in Fig. 3,

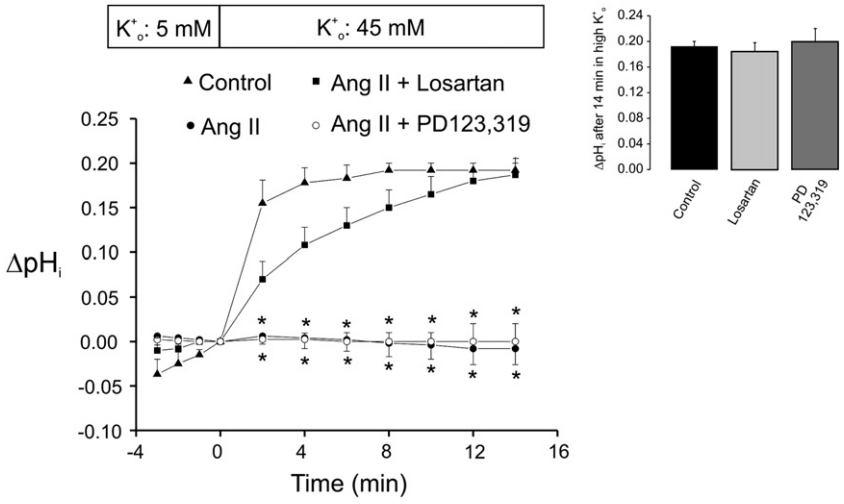

Fig. 2. Time course of the effect of Ang II on eNBC activity. Involvement of AT_1 and AT_2 receptors. Average data of pH_i alkalization induced by the hyperkalemic-induced depolarization of membrane potential in control (n = 6) and in the presence of 100 nM Ang II (n = 5), in the presence of Ang II plus the AT_1 receptor blocker losartan (1 μM; n = 4), or in the presence of Ang II plus the AT_2 receptor blocker PD123,319 (1 μM; n = 4). Data are expressed as an increase of pH_i units in comparison to the zero time point in high K^+ solution. * indicates $p < 0.05$ vs. control. Ang II was added to the bath solution 10 min before the potassium pulse. Losartan or PD123,319 was applied 20 min before the potassium pulse. Inset: Effect of losartan or PD123,319 on the basal rise of pH_i induced by the potassium pulse. The drugs were applied 20 min before the potassium pulse. Data are expressed as an increase of pH_i units after 14 min in high K^+ solution.

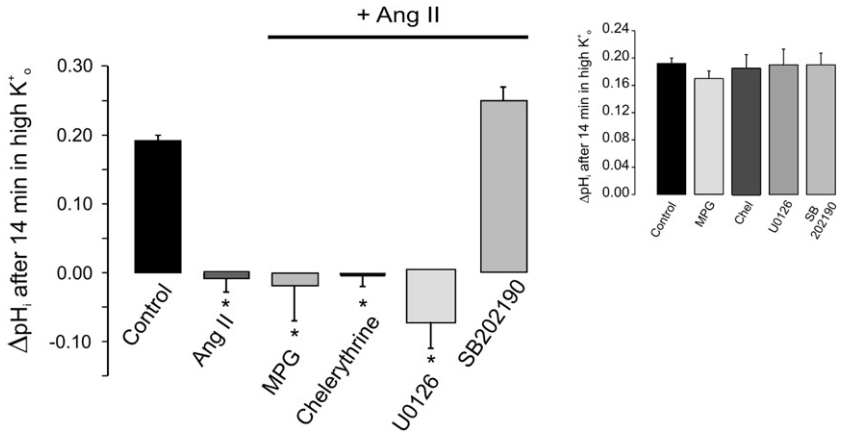

Fig. 3. Ang II inhibits eNBC via stimulation of the p38 kinase pathway. Average data of pH_i alkalization induced by the potassium pulse in control (n = 6), in the presence of 100 nM Ang II (n = 5) or in the presence of this hormone plus the ROS scavenger MPG (2 mM; n = 8), the PKC blocker chelerythrine (2 μM, n = 4), the ERK inhibitor U0126 (10 μM, n = 4) or the p38 kinase blocker SB202190 (10 μM, n = 5). Data are expressed as an increase of pH_i units after 14 min in high K^+ solution. * indicates $p<0.05$ vs. control. Ang II was added to the bath solution 10 min before the potassium pulse. MPG, chelerythrine, U0126 or SB202190 were applied 20 min before the potassium pulse. Inset: Effect of MPG, chelerythrine, U0126 or SB202190 on the basal rise of pH_i induced by the potassium pulse. The drugs were applied 20 min before the potassium pulse. Data are expressed as an increase of pH_i units after 14 min in high K^+ solution.

pre-treatment of the cells with SB202190 totally prevented the inhibitory effect of Ang II on eNBC mediated pH_i alkalization. In contrast, the pre-treatment with U0126 was unable to prevent the eNBC inhibition. These data indicate that Ang II-induced inhibition of eNBC activity requires the activation of p38 kinase, but is independent of the ERK1/2 pathway. Neither U0126 nor SB202190 affected the basal rise of pH_i induced by the depolarization of membrane potential (Fig. 3, inset).

3.4. Effect of Ang II on eNBC activity after intracellular acidification

As previously described, Ang II is known to accelerate the pH_i recovery after acidification both in rat and cat adult myocytes [18,19]. Regarding this, we have recently reported that Ang II-induced stimulation of sarcolemmal NBC activity is AT_1 receptor-mediated and requires the increment of ROS production and the activation of the ERK

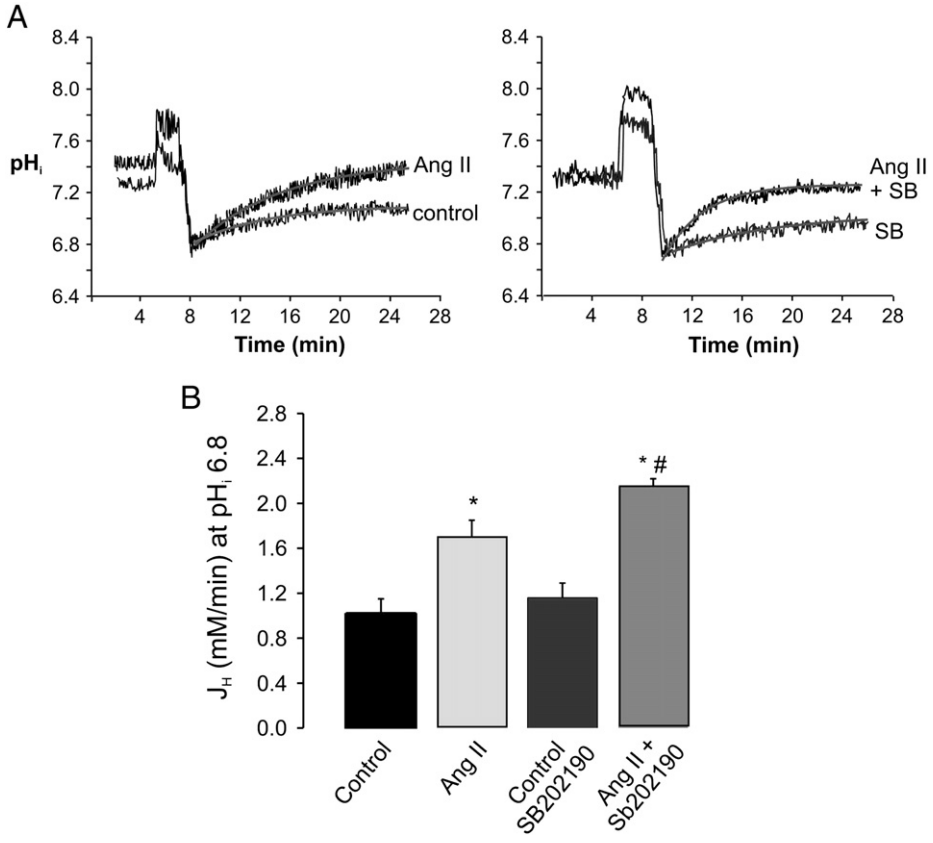

Fig. 4. Effect of p38 kinase inhibition on the Ang II-induced stimulation of total NBC activity after acidification. (Panel A) Representative traces of pH_i during the application of ammonium pulses in the absence and presence of 100 nM Ang II (left side) and in the absence and presence of 100 nM Ang II in the continuous presence of SB202190 (10 μM) (right side). (Panel B) Average J_H calculated at pH_i of 6.8 in control (n = 11), in the presence of 100 nM Ang II alone (n = 8), in the presence of SB202190 (10 μM, n = 5) or in the presence of Ang II plus SB202190 (n = 5). * and # indicate $p<0.05$ vs. control and Ang II alone, respectively. Ang II and SB202190 were applied 10 and 20 min before the ammonium pulse, respectively.

pathway [19]. We demonstrated that 100 nM Ang II induced an increase in J_H of approximately 70% at pH_i of 6.8 (Fig. 4). We hypothesized that if Ang II is inhibiting eNBC in a p38 kinase-dependent manner, after blockade of such pathway the Ang II-mediated increase in J_H will be greater. To confirm this hypothesis, we performed an ammonium prepulse in the presence of both Ang II and SB202190 in order to abrogate the negative pathway of Ang II. As expected, in this condition Ang II induced a higher increase in J_H (about 100% at pH_i of 6.8, Fig. 4) than it did without the p38 kinase inhibitor. All together these data allows us to suggest that Ang II inhibits eNBC through the p38 kinase pathway. Therefore, we can also suggest that the Ang II-induced increase in the total NBC activity previously reported (Fig. 4) might be due to an ERK1/2 and ROS dependent stimulation of nNBC, an effect that should overrule the inhibitory pathway mediated by eNBC blockade. Consistently, in the presence of NHE inhibition with HOE642, Ang II increased resting pH_i (0.24 ± 0.04, $n = 4$), likely due to nNBC stimulation overcoming eNBC inhibition.

3.5. Impact of the Ang II-induced eNBC inhibition in the cat ventricular AP

We have recently demonstrated that eNBC contributes to the normal configuration of the AP waveform of cat ventricular myocytes [11]. In that study, we reported that changing the myocyte bathing superfusate from a HCO_3^--free (HEPES-buffered) to a HCO_3^- containing solution at a constant pH_o, induces a 25% shortening of AP duration (APD; Fig. 5) consistent with the influx of HCO_3^- into the cell. These changes were sensitive to the extracellular concentration of HCO_3^- and were blunted by anionic blockade or by sodium deprivation [11], indicating that they are mediated by the repolarizing current generated by eNBC (I_{NBC}). Thus, we next evaluated the influence on APD of the inhibitory effect of Ang II on I_{NBC}. In perforated-patch experiments we measured APD in cat ventricular myocytes exposed to the change of the extracellular superfusate from HCO_3^--free (HEPES-buffered) to a HCO_3^- containing solution at a constant pH_o in the continuous presence of 100 nM Ang II. As shown in the representative traces of Fig. 5(A) and the average data of Fig. 5(B), no APD shortening was observed under these conditions, in contrast to the 25% APD shortening observed in the absence of the hormone (Fig. 5(B)). These results suggest that Ang II is able to block I_{NBC}. Note that Ang II induced a prolongation of APD before switching to the HCO_3^- containing solution ($26.1 \pm 9.3\%$, $n = 5$), as previously reported [29–31]. Also note that Ang II induced a hyperpolarization of resting membrane potential (RMP). This effect was observed in 4 out of 5 cells and its magnitude was variable (-2.7 ± 1.6 mV, $n = 5$). The effect of this hormone on RMP and/or ionic membrane currents is highly controversial and apparently depends on differences in concentration, cell types and/or species [32]. As previously reported in rabbit ventricular myocytes [33], an increase in the inward rectifier current (I_{K1}) after exposure to Ang II could explain the RMP hyperpolarization observed in the cat ventricular myocytes. However, other authors have found opposite effects of Ang II on I_{K1} of rat atrial myoctes [34] or mouse ventricular myocytes [35]. In addition, no effect of Ang II on I_{K1} of rat ventricular myocytes was also reported [36].

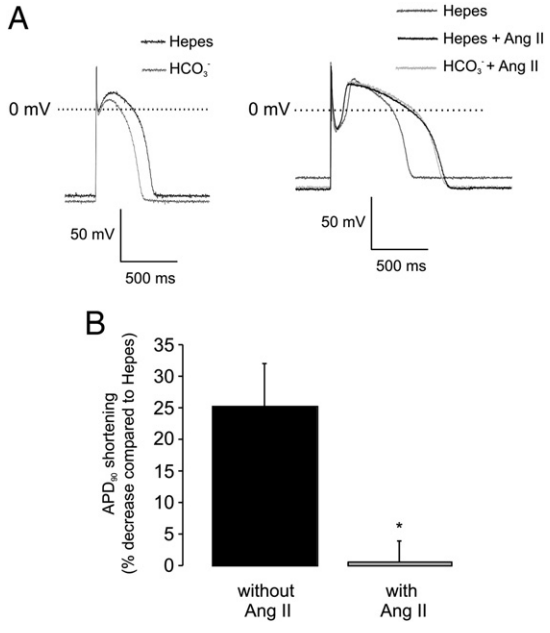

Fig. 5. Ang II cancels the influence of eNBC in AP configuration. (Panel A) Representative AP recordings performed with current-clamp in perforated-patch configuration obtained from a ventricular myocyte successively exposed 10 min to HEPES (bicarbonate-free solution) and 10 min to bicarbonate-containing solution (left side) and from another myocyte successively exposed 10 min to HEPES (bicarbonate-free solution), 15 min to HEPES in the presence of 100 nM Ang II and 10 min to bicarbonate-containing solution in the continuous presence of Ang II (right side). (Panel B) Average APD_{90} after 10 min of switching the extracellular solution from HEPES to bicarbonate in the absence ($n = 7$) and presence of 100 nM Ang II ($n = 5$). The values were expressed as percent change with respect to the value in HEPES measured immediately before the switch to bicarbonate. * indicates $p < 0.05$ vs. without Ang II.

4. Discussion

The main finding of this study is that Ang II exerts an inhibitory effect on the cardiac eNBC and that this effect involves the AT_1 receptor activation which depends on the stimulation of the p38 kinase pathway. Previous evidence has indicated that Ang II stimulates total cardiac NBC activity [16,18,19]. We have recently reported that this effect is mediated by the production of ROS and the activation of the MAPK ERK [19]. However, we have not identified the NBC isoform implicated in such an effect. Taking into account the results of the present work, we can speculate that the NBC-mediated enhanced pH_i recovery from acidification induced by Ang II previously detected might be due to the activation of nNBC. A schematic drawing of the proposed mechanisms of action of Ang II on NBC is presented in Fig. 6, in which results, conclusions and speculations derived from the present and previous studies about this matter are summarized.

Although functional studies have suggested the presence of nNBC in cardiac ventricular myoctes [2,3,10], the expression of NBC3 (only nNBC isoform identified in the heart) in these cells is controversial [10,37–39]. Its presence in rat and human ventricular myocardium [10,37] but not in

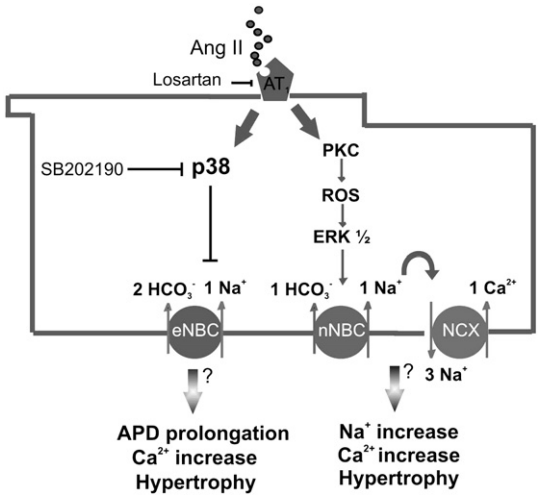

Fig. 6. Schematic diagram showing the proposed mechanisms involved in the Ang II-induced actions on cardiac NBC. Ang II, binding to AT_1 receptors, exerts an opposite effect on NBC isoforms via the activation of two parallel kinase pathways: the ERK 1/2 and p38. nNBC stimulation is mediated by ROS and ERK 1/2, and p38 kinase mediates the Ang II-induced eNBC inhibition. These findings could be relevant in pathologies, such as hypertrophy and heart failure, in which it has been reported as either intracellular calcium overload or potential action prolongation, that could be explained by the nNBC stimulation and the eNBC inhibition, respectively.

mouse ventricular cardiomyoctes [39] has been reported. In preliminary experiments we were able to detect a band of approximately 130 kDa, consistent with the molecular weight of NBC3 [40], in western blots of cat ventricular myocardium and HEK293 cells transfected with human NBC3 as positive control (unpublished data), suggesting that this electroneutral isoform of NBC could be indeed responsible for the stimulation of the NBC-mediated pH_i recovery from acidosis induced by Ang II via the ERK pathway (Fig. 6). Nevertheless, as ERK is not only up-regulated by Ang II but also by acidosis [18,41], we cannot discard the possibility that eNBC might also be activated by this pathway during the acidification produced by the ammonium pulse.

The membrane potential depolarization induced by high extracellular potassium used herein to isolate eNBC activity is likely to promote Ca^{2+} influx through L-type Ca^{2+} channels and affect the S0859-sensitive eNBC-mediated pH_i changes observed during this protocol. However, the absence of effects of Ca^{2+} and/or the Ca^{2+}-calmodulin dependent kinase II on rat cardiac NBC activity [7] has been previously reported. Furthermore, although intracellular calcium measurements were not performed in these cells herein, it seems unlikely that substantial increases in intracellular calcium are taking place during the potassium pulse in our experiments because, despite the fact that the myocytes were continuously paced at 0.5 Hz, no twitch contractions and/or significant contracture were observed during this methodological approach in any of the cells studied (as an example, a representative continuous recording of sarcomere shortening before, during and after the potassium pulse is included in the Supplementary material). The expected abolition of excitability due to the depolarized membrane potential might be accompanied by minimization of calcium influx through inactivated calcium channels. On the other hand, it is also possible that the high K^+ depolarization is not sufficient to reach the reversal potential of the sodium/calcium exchanger, avoiding the operation of the calcium influx mode of this transporter. However, these indirect evidences do not allow us to completely discard some contribution of calcium to the eNBC-mediated pH_i changes observed during the potassium pulse.

Although we did not identify the electrogenic isoform involved (NBC1 or NBC4), in the present study we demonstrated for the first time that general cardiac eNBC activity is in fact abolished by Ang II. In agreement with these results, it has been previously reported that renal NBC1 is not affected or inhibited by Ang II at concentrations higher to 10 nM in an arachidonic acid-dependent way [42]. Consistently, it has been previously reported that p38 kinase is related with the arachidonic acid pathway and its metabolites in several tissues [43–46]. Thus, further investigations are needed to evaluate the participation of arachidonic acid in the Ang II pathway in ventricular myocytes.

The Ang II-induced inhibition of eNBC observed in the present work was not cancelled by the PKC inhibitor, chelerythrine, the ERK1/2 inhibitor, U0126 or scavenging ROS with MPG, indicating that none of these molecules is involved in the intracellular pathway of the inhibitory effect of Ang II. These data also allow us to suggest that under our conditions the eNBC inhibition induced by Ang II after the stimulation of p38 kinase does not require the increase in the intracellular concentration of ROS. Although several studies place the increase in ROS production between the cardiac AT_1 receptor activation and the stimulation of p38 kinase [47–50], other works demonstrated ROS-independent Ang II-induced activation of this kinase [51,52]. The possibility that distinct p38 kinase isoforms are activated by oxidative stress-dependent and -independent pathways might be also considered [53].

It is well-known that Ang II modulates the properties of ion channels, leading to cardiac APD prolongation [31,35,54,55]. It has been reported that Ang II both inhibits repolarizing currents, as I_{K1}, I_{Kr} and I_{to} [35,55–57], and stimulates depolarizing currents as those carried by L-type calcium channels [20,58]. We demonstrated herein that in the presence of Ang II the eNBC-mediated APD shortening previously determined [11] does not occur, indicating that the hormone is abrogating the activity of the transporter. Thus, it is possible to speculate that this inhibition of eNBC by Ang II could be contributing to the APD prolongation induced by this hormone.

The contribution of NBC to the rapid restoration of normal pH_i upon reperfusion after ischemic acidosis is widely recognized [59–62]. However, since NBC is a cellular "Na^+-loading" mechanism, it is important to mention that its participation during reperfusion might contribute to Na^+ and Ca^{2+} overload and arrhythmia [62,63] Consistent with this hypothesis, Khandoudi et al. [37] reported that blockade of rat cardiac NBC1 during reperfusion results in cardioprotection. Thus, we can speculate that the Ang II-induced inhibition of eNBC detected herein would also carry beneficial effects under this pathological state. However, at the same time, the potential Ang II-induced stimulation of nNBC activity [19] would, in contrast, be associated to enhanced deleterious effects during reperfusion.

Similar paradigmatic speculations about the opposite roles of the effects of Ang II on nNBC and eNBC could be made with other pathological states such as cardiac hypertrophy and heart failure, which are cardiac diseases associated to elevated Ang II concentration [64,65], enhanced expression of NBC1 and/or NBC3 [37,66] and Na^+ overload [63]. However, in cardiac hypertrophy or heart failure, the potential attenuation of Na^+ overload that might be produced by the inhibition of eNBC in the presence of Ang II could be overruled by the deleterious effects that might be carried by the prolongation of APD induced by blockade of the anionic current generated by this transporter. At cellular level, prolongation of APD is consistently observed in many experimental models of cardiac hypertrophy and failure. It is also known that this can lead to QT prolongation in the electrocardiogram that in turn promotes an arrhythmogenic state which contributes to an increase in cardiovascular mortality [29]. Lebeche et al. [67] have reported that APD prolongation promotes an increase in intracellular calcium concentration. Since this ion is an essential cofactor in the activation of several hypertrophic signaling pathways, it is conceivable that this APD prolongation might be a cause and not a consequence of cardiac hypertrophy (Fig. 6). Nevertheless, further research is needed to assess the exact pathophysiological relevance of the Ang II-induced stimulation and inhibition of nNBC and eNBC, respectively.

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