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# A low dose of angiotensin II increases inotropism through activation of reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange by endothelin release

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## Abstract

**Objective:** This work was aimed to prove that release/formation of endogenous endothelin acting in an autocrine/paracrine fashion contributes to the increase in contractility promoted by a low dose of angiotensin II. **Methods:** Isolated cat papillary muscles were used for force,  $\text{pH}_i$ ,  $[\text{Na}^+]_i$  and  $[\text{Ca}^{2+}]_i$  measurements and isolated cat myocytes for patch-clamp experiments. **Results:** In papillary muscles, 1.0 nmol/l angiotensin II increased force by  $23 \pm 2\%$  ( $n=4$ ,  $P<0.05$ ),  $[\text{Na}^+]_i$  by  $2.2 \pm 0.2$  mmol/l ( $n=4$ ,  $P<0.05$ ), and peak (but not diastolic)  $\text{Ca}^{2+}$  from  $0.674 \pm 0.11$  to  $0.768 \pm 0.13$   $\mu\text{mol/l}$  ( $n=4$ ,  $P<0.05$ ), without affecting  $\text{pH}_i$ . Force and  $[\text{Na}^+]_i$  increase were abolished by inhibition of the  $\text{Na}^+/\text{H}^+$  exchanger (NHE) with the inhibitor HOE642, blockade of endothelin receptors with the nonselective antagonist TAK044 and by inhibition of the endothelin-converting enzyme with phosphoramidon. Force but not  $[\text{Na}^+]_i$  increase was abolished by inhibition of reverse  $\text{Na}^+/\text{Ca}^{2+}$  exchange (NCX) with the inhibitor KB-R7943. Similar increase in force ( $21 \pm 2\%$ ,  $n=4$ ,  $P<0.05$ ) and in  $[\text{Na}^+]_i$  ( $2.4 \pm 0.4$  mmol/l,  $n=4$ ,  $P<0.05$ ) that were also suppressed by TAK044 and HOE642 were induced by exogenous 5.0 nmol/l endothelin-1. KB-R7943 reverted the endothelin-1 effect on force but not on  $[\text{Na}^+]_i$ . In isolated myocytes, exogenous endothelin-1 dose-dependently increased the NCX current and shifted the NCX reversal potential ( $E_{\text{NCX}}$ ) to a more negative value ( $\Delta E_{\text{NCX}}$ :  $-10 \pm 3$  and  $-17 \pm 5$  mV, with 1 and 10 nmol/l endothelin-1, respectively,  $n=12$ ). The latter effect was prevented by HOE642. **Conclusion:** Taken together, the results indicate that a low dose of angiotensin II induces release of endothelin, which, in autocrine/paracrine fashion activates the  $\text{Na}^+/\text{H}^+$  exchanger, increases  $[\text{Na}^+]_i$  and changes  $E_{\text{NCX}}$ , promoting the influx of  $\text{Ca}^{2+}$  that leads to a positive inotropic effect (PIE).

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**Keywords:** Angiotensin; Endothelins; Ion transport; Na/Ca-exchanger; Na/H-exchanger; Cat papillary muscles; Isolated cat myocytes; E–C coupling

## 1. Introduction

Many of the effects previously attributed to angiotensin II (Ang II) have been shown to be mediated by autocrine/paracrine release/formation of endothelin (ET) [1–12]. Ang II releases/forms ET from many different types of tissue [1–12] including the heart [3–5]. In the heart, the mechanism has been associated with cardiac hypertrophy [3,4]

and fibrosis [5]. However, whether or not the release/formation of ET is also involved in the positive inotropic effect (PIE) of Ang II has not been explored. In previous studies, and based on pharmacological interventions, we suggested the existence of an autocrine/paracrine mechanism by which myocardial stretch induces the release of preformed Ang II, which will increase the release/formation of ET [13–15]. Then ET, by activating the  $\text{Na}^+/\text{H}^+$  exchanger (NHE), will increase  $[\text{Na}^+]_i$  and promote  $\text{Ca}^{2+}$  influx through the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX). Although each step of this chain of events was pharmacologically confirmed [13–15], evidence that exogenously applied Ang II releases ET and increases myocardial contractility is still lacking.

The aim of this study was to prove that a low dose of Ang II promotes a PIE by increasing the release/formation

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of ET by myocardial cells and to identify the subsequent underlying mechanism.

## 2. Materials and 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. Multicellular preparations

As previously described [15], cat papillary muscles from the right ventricle were mounted in a perfusion chamber placed on the stage of an inverted microscope for force and epifluorescence measurements and superfused at constant rate (5 ml/min) with a  $\text{CO}_2/\text{HCO}_3^-$ -buffered solution containing (mmol/l) 128.3 NaCl, 4.5 KCl, 1.35  $\text{CaCl}_2$ , 20.23  $\text{NaHCO}_3$ , 1.05  $\text{MgSO}_4$ , 11.0 glucose, equilibrated with 5%  $\text{CO}_2/95\% \text{O}_2$  at 30 °C (solution pH ~ 7.39). To avoid the

contribution of catecholamine released by nerve endings in the PIE of Ang II [16], prazosin plus atenolol (1.0  $\mu\text{mol/l}$  each) were used. The muscles were paced at 0.2 Hz at a voltage 10% over threshold, and isometric contractions were recorded. Cross-sectional area (0.75 of the product of thickness by width) was used to normalise the force records obtained with a silicon strain gauge (model AEM 801, SensoNor). After determining slack length, each muscle was progressively stretched to the length at which twitch-developed force was maximal ( $L_{\text{max}}$ ). Then, they were shortened to obtain the 95% of the maximal twitch force (length that approximated 98% of  $L_{\text{max}}$ ) and maintained at this length throughout the rest of the experiment. Changes in  $[\text{Na}^+]_i$  and  $\text{pH}_i$  were performed by epifluorescence with sodium-binding benzofuran isophthalate (SBFI, AM form) or 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF, AM form), respectively, as previously described [15]. Briefly, the esters were prepared as 1 mmol/l solution in dimethylsulfoxide (DMSO); this solution was mixed with Pluronic F-127 (20% wt./wt. in DMSO) at a ratio 4:1 to help disperse the indicators in the loading medium. Loading time

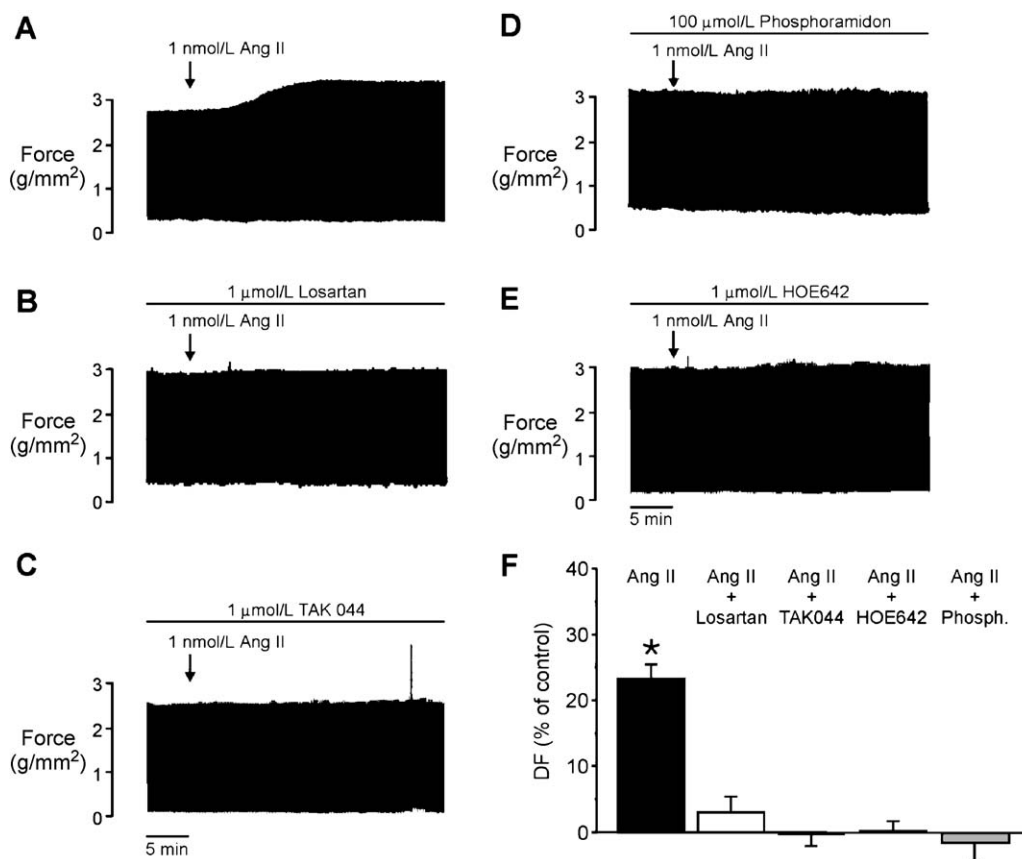


Fig. 1. Inotropic effect of 1 nmol/l Ang II on contractility of isolated cat papillary muscles. Typical force record showing the positive inotropic effect promoted by Ang II (A). Blocking the  $\text{AT}_1$  receptors with losartan (B), nonspecific blockade of ET receptors with TAK044 (C), endothelin-converting enzyme inhibition with phosphoramidon (D) or NHE-1 blockade with HOE642 (E) abolished the increase in contractility. Overall changes in developed force (DF) at the end of 30 min of incubation with the peptide, expressed as percent of baseline (pre-Ang II) value (F,  $n=4$  each). These results suggest that exogenous Ang II, through its  $\text{AT}_1$  receptors, promotes release/formation of ET that activates the NHE-1 and induces the increase in force. \*indicates  $P < 0.05$  vs. pre-Ang II value.

lasted 30 min with BCECF (final concentration 5  $\mu\text{mol/l}$ ) and 2 h with SBFI (final concentration 10  $\mu\text{mol/l}$ ) at room temperature (22–24 °C), followed in both cases by a 30–60-min washout to allow the cleavage of the AM part of the dyes by intracellular esterase. At the end of each experiment, BCECF emitted fluorescence was calibrated in vivo by the high  $\text{K}^+$ -nigericin method [13–15]. Calibration of SBFI fluorescence was made according to Harootunian et al. [17] with 2.0  $\mu\text{mol/l}$  gramicidin, 5.0  $\mu\text{mol/l}$  monensin and 0.05 mmol/l ouabaine. Calibration media containing various  $\text{Na}^+$  concentrations were made from appropriate mixtures of high  $\text{Na}^+$  and high  $\text{K}^+$  solutions. In the case of Fura-2 AM, the muscles were incubated with the dye for 1.5 h (final concentration 5  $\mu\text{mol/l}$ ) followed, by a 30–60-min washout. Contribution of fluorescence arising from dye in endothelial cells was disregarded since no significant changes in the absolute level of fluorescence was detected during prolonged ( $\sim 1.5$  h) monitoring after inducing the selective endothelial loss of the indicator by adding 100 nmol/l ionomycin at the end of each experiment [18].  $[\text{Ca}^{2+}]_i$  was calculated by the standard equation  $[\text{Ca}^{2+}]_i = K_d' (R - R_{\min}) / (R_{\max} - R)$ . In vivo calibration of Fura-2 signals gave values of 2.63, 2.11 and 0.511 for  $K_d'$ ,  $R_{\max}$  and  $R_{\min}$ ,

respectively. For all indicators, corresponding background fluorescence levels at each wavelength was subtracted from the respective signal before rationing.

## 2.2. Cell isolation

Cat cardiac myocytes were isolated as previously described [19] and were placed in a perfusion chamber superfused at a constant rate of 1.5 ml/min at 30 °C. The patch-clamp whole-cell configuration technique was used for voltage-clamp recordings. According to the voltage protocol described by Zhang et al. [20], whole-cell currents were evoked by descending ramps from +80 to  $-120$  mV ( $dV/dt = 0.4$  mV/ms), applied from a holding potential of  $-80$  mV. Prior to the descending ramp, the membrane potential was held at +80 mV for 30 ms in order to avoid current contamination during the ramp with any residual uncompensated capacitative current. Interpulse interval was 10 s.

## 2.3. Isolation NCX current ( $I_{\text{NCX}}$ )

The  $I_{\text{NCX}}$  was isolated as previously described [21]. The NCX blocker  $\text{NiCl}_2$  ( $\text{Ni}^{2+}$ , 10 mmol/l) was added to the

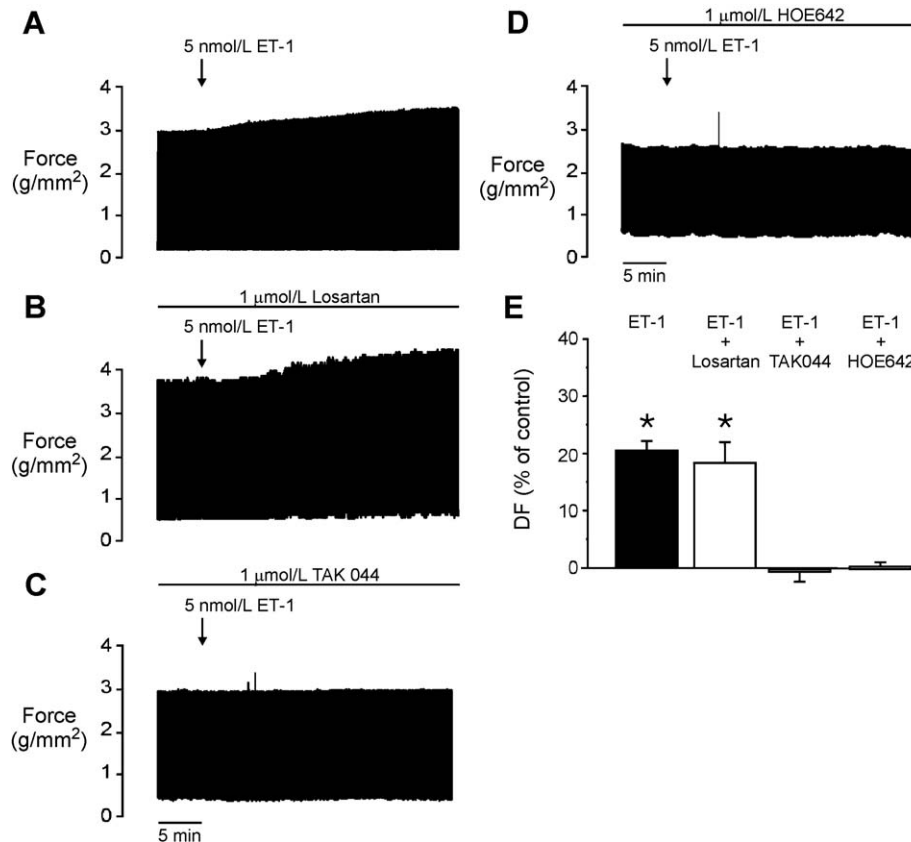


Fig. 2. Inotropic effect of 5 nmol/l ET-1. As in Fig. 1, typical force records of the effect of ET-1 alone (A) or after different pharmacological interventions (B–D) are shown. Overall values of DF, expressed as percent of baseline (pre-ET-1) value, after 30 min (E,  $n = 4$  each). The results show that the ET-1 effect on NHE-1 activity is mediated by its own receptors, since it was abolished by TAK044 but not by losartan. The fact that TAK044 abolished the effects of Ang II (Fig. 1) indicates that Ang II acts through ET but not ET through Ang II. \* Indicates  $P < 0.05$  vs. pre-ET-1 value.

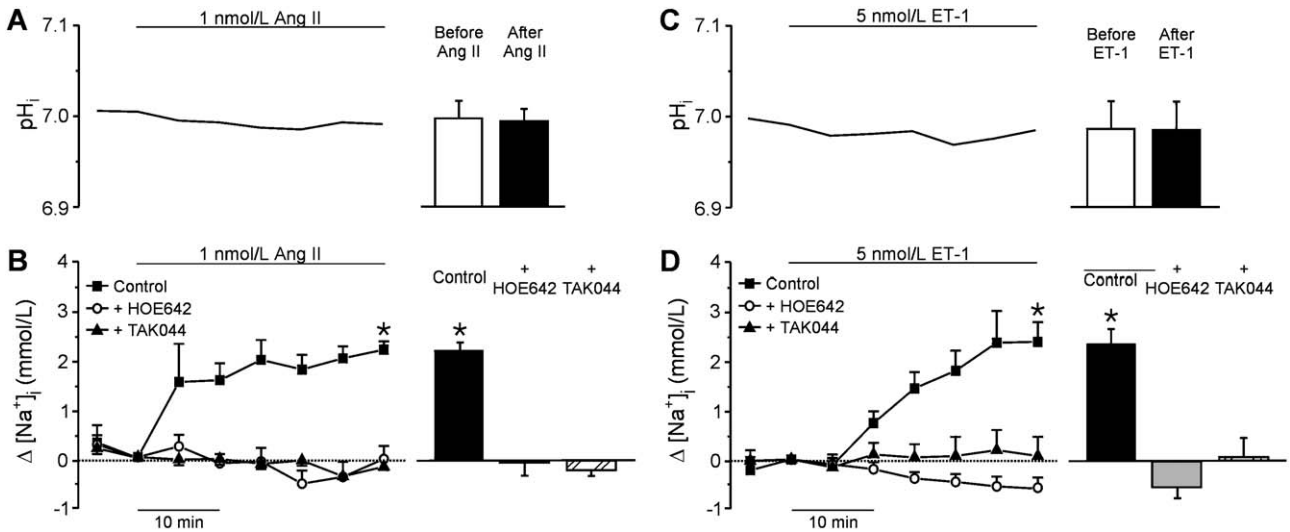


Fig. 3. Ang II and ET-1 induced NHE-1 activation. (A) Typical experiment and averaged results ( $n=4$ ) of the effect of 1.0 nmol/l Ang II on  $pH_i$ . (B) Time course of  $[Na^+]_i$  changes induced by Ang II and averaged values after 30 min, in control and after NHE-1 inhibition or ET receptors blockade. (C and D) Same as (A) and (B), respectively, but after addition of a dose of exogenous ET-1 (5.0 nmol/l) equipotent to 1.0 nmol/l Ang II. These results show that despite the lack of  $pH_i$  change, there is an Ang II-induced NHE-1 stimulation detected by the increase in  $[Na^+]_i$  that requires available ET receptors and that this effect can be mimicked by an equipotent dose of exogenous ET-1. \* Indicates  $P < 0.05$  vs. pre-Ang II (B) or pre-ET-1 (D) value.

extracellular solution at the end of each experiment to obtain the  $Ni^{2+}$ -sensitive currents, representing  $I_{NCX}$ . For each cell, capacitive current was recorded to normalise the currents for cell capacitance. Averaged cell capacitance was  $131 \pm 5$  pF ( $n=20$ ).

#### 2.4. Pharmacological interventions

The effect of Ang II or ET-1 was assessed in the absence and presence of 1.0  $\mu\text{mol/l}$  HOE642 to inhibit the cardiac  $Na^+/H^+$  exchanger (NHE-1 isoform); 1.0  $\mu\text{mol/l}$  losartan, to

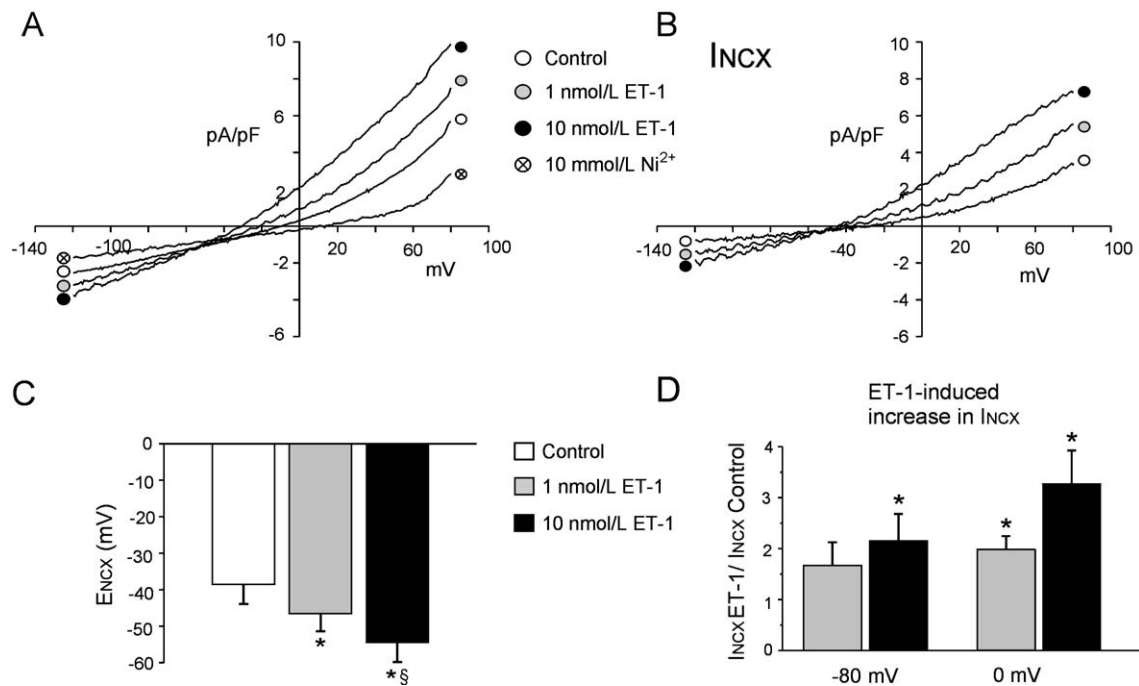


Fig. 4. Effects of ET-1 on  $I_{NCX}$ . Representative traces of whole-cell currents evoked by descending ramps recorded in a cat ventricular myocyte before (Control) and after successive additions of 1.0, 10.0 nmol/l ET-1 and 10.0 mmol/l  $Ni^{2+}$  to the bath solution (A).  $Ni^{2+}$ -sensitive currents, representing  $I_{NCX}$ , obtained by subtracting the currents recorded in  $Ni^{2+}$  to those of control and ET-1 (B). Average values of  $E_{NCX}$  obtained from the  $Ni^{2+}$ -sensitive currents recorded in 12 myocytes are shown in (C). Bars compare the ratio between control  $I_{NCX}$  and that in the presence of ET-1 at potentials of 0 and  $-80$  mV (D). Note that ET-1 dose-dependently increased both outward and inward  $I_{NCX}$  and shifted the  $E_{NCX}$  to a more negative value. \* Indicates  $P < 0.05$  vs. control. § Indicates  $P < 0.05$  vs. 1.0 nmol/l ET-1.

block the AT<sub>1</sub> receptors; 1.0 μmol/l TAK044 for nonspecific ET receptors blockade or 5.0 μmol/l KB-R7943, to inhibit the reverse mode of the NCX. The effect of Ang II was also assessed in the presence of 100 μmol/l phosphoramidon to inhibit the endothelin-converting enzyme. In all cases, the drugs were applied ~ 20 min before Ang II or ET-1, and during this period, developed force (DF) did not change by more than 3.0%. It was carefully checked that DF was at steady state before adding any peptide.

2.5. Statistics

Data are expressed as mean ± S.E.M. Paired or unpaired Student’s *t*-test or one-way ANOVA followed by Student–Newman–Keuls were used as appropriate. A *P* < 0.05 was considered to indicate significant differences.

3. Results

Fig. 1A shows the effect of 1.0 nmol/l Ang II on the contractile behaviour of a cat isolated papillary muscle. The concentration was selected to induce ~ 20% increase in contractility. The averaged increase in force after 30 min of Ang II was 23 ± 2% of control (*n* = 4, *P* < 0.05, Fig. 1F).

This effect was completely abolished by either AT<sub>1</sub> receptor blockade with losartan (Fig. 1B and F), nonspecific ET receptor blockade with TAK044 (Fig. 1C and F), endothelin-converting enzyme inhibition with phosphoramidon (Fig. 1D and F) or NHE-1 blockade with HOE642 (Fig. 1E and F), giving support to the hypothesis that Ang II promotes, at least at this dose and through AT<sub>1</sub> receptors, the release/formation of ET, which acting in an autocrine or paracrine fashion activates the NHE-1 and determines the contractile changes. A dose of exogenous ET-1 (5.0 nmol/l) almost equipotent to 1.0 nmol/l Ang II (see Fig. 2A, typical experiment) seems to utilize a pathway similar to that of the octapeptide, since its PIE (21 ± 2%, *n* = 4, *P* < 0.05, Fig. 2A and E) was cancelled by TAK044 (Fig. 2C and E) and by HOE642 (Fig. 2D and E). The PIE of ET-1 was not prevented by AT<sub>1</sub> receptors blockade with losartan (force increased by 18 ± 4%, *n* = 4, *P* < 0.05, Fig. 2B and E), which allows the conclusion that Ang II acts through ET but not ET through Ang II.

The enhanced NHE-1 activity caused by ET, either when released in response to Ang II or when exogenously applied, could potentially increase contractility by a myofilament-sensitising effect due to a rise in p*H*<sub>i</sub> and/or to an increase in [Ca<sup>2+</sup>]<sub>i</sub> secondary to a rise in [Na<sup>+</sup>]<sub>i</sub>. Consistent with our previous results [22–24], no significant change

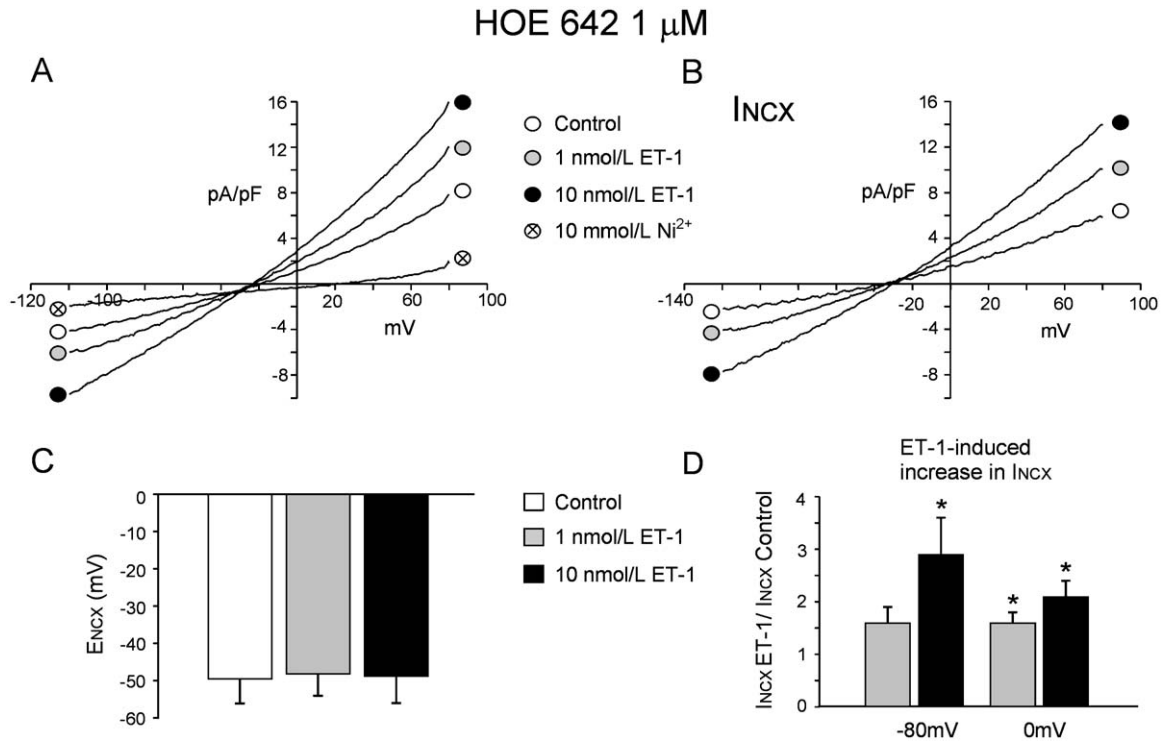


Fig. 5. Effects of ET-1 on *I*<sub>NCX</sub> in the presence of NHE-1 inhibition. Representative traces of whole-cell currents before (Control) and after successive addition of 1.0, 10.0 nmol/l ET-1 and 10.0 mmol/l Ni<sup>2+</sup> in the continuous presence of 1.0 μmol/l HOE 642 (A). (B) Ni<sup>2+</sup>-sensitive currents, obtained from currents of panel (A). Average values of *E*<sub>NCX</sub> obtained from the Ni<sup>2+</sup>-sensitive currents recorded in eight myocytes (C). Bars compare the ratio between control *I*<sub>NCX</sub> and that in the presence of ET-1 at potentials of 0 and -80 mV (D). Note that NHE-1 inhibition prevented the *E*<sub>NCX</sub> negative shift, in agreement with the fact that no rise in [Na<sup>+</sup>]<sub>i</sub> was detected when NHE-1 activity was inhibited, as was shown in Fig. 3. \* Indicates *P* < 0.05 vs. control.



in  $pH_i$  was detected after addition of Ang II or ET-1 (Fig. 3A and C, respectively), thus rejecting  $pH_i$  as a possible cause of the PIE. The lack of changes in  $pH_i$  after NHE-1 activation by Ang II or ET-1 is not a novel finding. We have previously shown that in the presence of bicarbonate, both peptides simultaneously activate the alkalinising NHE-1 and the acidifying  $Na^+$ -independent  $Cl^-/HCO_3^-$  exchanger, thus compensating changes in  $pH_i$  [22,23]. Instead, the activation of the NHE-1 was evidenced by an increase in  $[Na^+]_i$  that after 30 min was  $2.2 \pm 0.2$  mmol/l over control ( $n=4$ ,  $P<0.05$ ) for Ang II and  $2.4 \pm 0.4$  mmol/l ( $n=4$ ,  $P<0.05$ ) for ET-1 (Fig. 3B and D, respectively). In addition, the rise in  $[Na^+]_i$  caused by Ang II was accompanied by an increase in peak systolic intracellular  $Ca^{2+}$  from  $0.674 \pm 0.11$  to  $0.768 \pm 0.13$   $\mu\text{mol/l}$  after Ang II ( $n=4$ ,  $P<0.05$ ), without significant changes in diastolic  $Ca^{2+}$  ( $0.310 \pm 0.03$  vs.  $0.317 \pm 0.03$   $\mu\text{mol/l}$ ). Note that the cancellation of the PIE by NHE-1 inhibition or ET receptor blockade (Figs. 1 and 2) concurred with the prevention of Ang II and ET-1-induced increase in  $[Na^+]_i$  (Fig. 3B and D).

So far, the results clearly link the ET-mediated NHE-1 activation with the rise in  $[Na^+]_i$  and this rise with the PIE. A plausible explanation for this link (indirectly supported by previous experiments [13,14]) would be that the rise in  $[Na^+]_i$  may produce a negative shift in the reversal potential of the NCX ( $E_{NCX}$ ), thus allowing the NCX to operate in reverse mode for more time during the action potential and promoting cell  $Ca^{2+}$  influx. To analyse this hypothesis, the effect of ET-1 on  $E_{NCX}$  were examined in isolated myocytes. Fig. 4A depicts representative traces of whole-cell

currents evoked by voltage-clamped descending ramps before and after ET-1. Current density was dose-dependently increased by the peptide. The recorded currents were mostly but not totally  $I_{NCX}$  since a remnant current was present after NCX blockade with  $Ni^{2+}$ . This remnant  $Ni^{2+}$ -insensitive current was not affected by ET-1 (at 60 mV:  $0.52 \pm 0.18$ ,  $0.52 \pm 0.17$  and  $0.62 \pm 0.19$  pA/pF, in control, 1.0 and 10.0 nmol/l ET-1, respectively,  $n=6$ ) as it was also reported by Zhang et al. [20] Fig. 4B shows the effect of ET-1 on the  $Ni^{2+}$ -sensitive currents (representing  $I_{NCX}$ ) obtained by subtracting the currents recorded in  $Ni^{2+}$  to those recorded in control and in the presence of ET-1. Averaged values of  $E_{NCX}$  before and after myocyte exposure to 1.0 and 10.0 nmol/l ET-1 are shown in Fig. 4C. ET-1 produced a clear negative shift of  $E_{NCX}$  ( $\Delta E_{NCX}$ : 1.0 nmol/l ET-1 =  $-10 \pm 3$  mV, 10.0 nmol/l ET-1 =  $-17.1 \pm 5.1$  mV,  $n=12$ ,  $P<0.05$ ) and also induced an increase in both outward and inward  $I_{NCX}$ , as can be appreciated by the  $I_{NCX}$  recorded at 0 and  $-80$  mV, respectively (Fig. 4D). In agreement with previous reports [20,25], this increase in  $I_{NCX}$  demonstrates a direct stimulation of the NCX by ET-1.

The ET-1-induced negative shift in  $E_{NCX}$  is likely to be the result of the NHE-1 mediated increase in  $[Na^+]_i$  induced by the peptide, since it was cancelled by preincubation with HOE642. Representative current traces are shown in Fig. 5A and B. HOE642 did not affect basal  $E_{NCX}$  value but totally cancelled the negative shift (Fig. 5C;  $\Delta E_{NCX}$ : 1.0 nmol/l ET-1 =  $2.1 \pm 3$  mV, 10.0 nmol/l ET-1 =  $1.7 \pm 3.5$  mV,  $n=8$ ). HOE642 did not prevent the ET-1-induced increase in outward and inward  $I_{NCX}$  (Fig. 5D), confirming

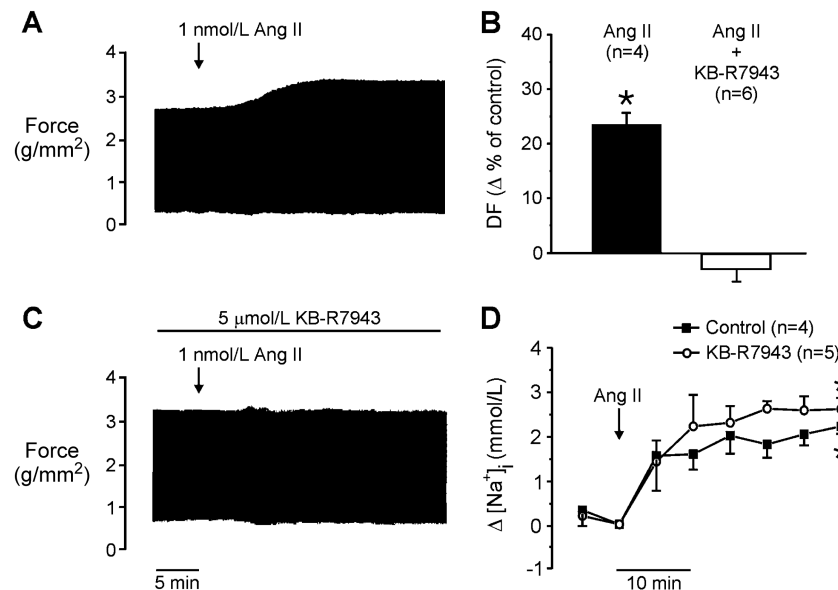


Fig. 6. Effects of reverse mode NCX inhibition on the inotropic response to Ang II. The typical force record of Fig. 1 is reproduced here (A) to facilitate comparisons. In the presence of KB-R7943, inhibitor of the reverse mode NCX, the contractile effect of Ang II was canceled [(C) typical force record; (B) averaged results after 30 min of Ang II]. Note that although KB-R7943 prevented the increase in DF, it did not prevent or even slightly increased the rise in  $[Na^+]_i$  (D), giving support to the idea that the exchange between  $Na^+$  and  $Ca^{2+}$  is blocked. \* Indicates  $P<0.05$  vs. pre-Ang II value.

that it should be due to a direct effect of the peptide on the NCX (probably by PKC-dependent protein phosphorylation [25]).

Although we cannot rule out the possibility that in these experiments, the activation of the NHE-1 could increase  $\text{pH}_i$  and induce HOE642-sensitive changes in membrane currents, the negative shift in the  $E_{\text{NCX}}$  described here is characteristic of a change in  $[\text{Na}^+]_i$ , which, on the other hand, will favour the reverse working mode of the NCX. It is important to note that the negative  $E_{\text{NCX}}$  shift took place despite the cells being dialyzed with a pipette solution of constant  $[\text{Na}^+]_o$ , which very likely clamps bulk  $[\text{Na}^+]_i$ . However,  $\text{Na}^+$  accumulation might occur at a certain micro-domain influencing  $E_{\text{NCX}}$  to explain the present results. In this regard, co-localization of NHE-1 and NCX was previously reported [26,27].

The idea that a negative  $E_{\text{NCX}}$  shift driving the reverse mode NCX would contribute to the PIE induced by a low dose of Ang II (or its proposed endogenous mediator ET) is further supported by the experiments shown in Figs. 6 and 7. Blockade of the reverse mode NCX with KB-R7943 fully prevented the increase in contractility induced by 1 nmol/l Ang II (Fig. 6A–C). At the same time, it did not affect or even slightly increased the rise in  $[\text{Na}^+]_i$  (Fig. 6D), giving support to the idea that the  $\text{Na}^+$  for  $\text{Ca}^{2+}$  exchange was blocked. Interestingly, addition of KB-R7943 at the steady state of the PIE due to 5 nmol/l ET-1 reverted the effect (Fig. 7A and B), while  $[\text{Na}^+]_i$  continued rising (Fig. 7C). Finally, although it can be argued that KB-R7943 is not a specific blocker of the reverse mode of the NCX, Fig. 8 shows that the concentration used here did not affect basal contractility (panels A and B) or the  $\sim 20\%$  increase in contractility

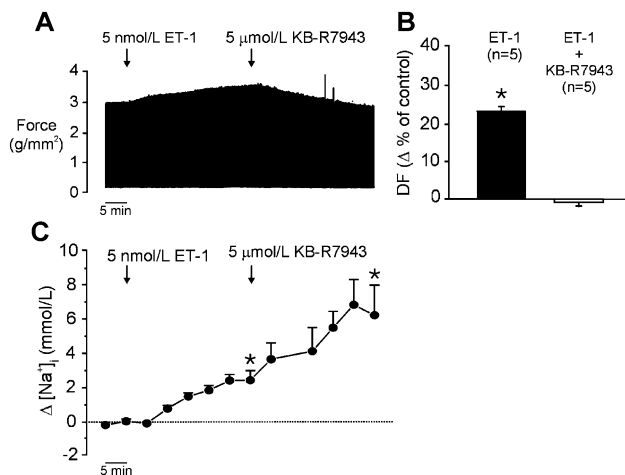


Fig. 7. Effects of reverse mode NCX inhibition on the inotropic response to ET-1. Addition of KB-R7943 after full development of the PIE due to exogenous ET-1 reverted the effect [(A) typical force record; (B) averaged results after 30 min of ET-1] but did not prevent the increase in  $[\text{Na}^+]_i$  that continued rising (C). \* Indicates  $P < 0.05$  vs. pre-ET-1 value. For the sake of clarity, asterisks were only placed at 30 min of ET-1 and at 30 min of ET-1 + KB-R7943.

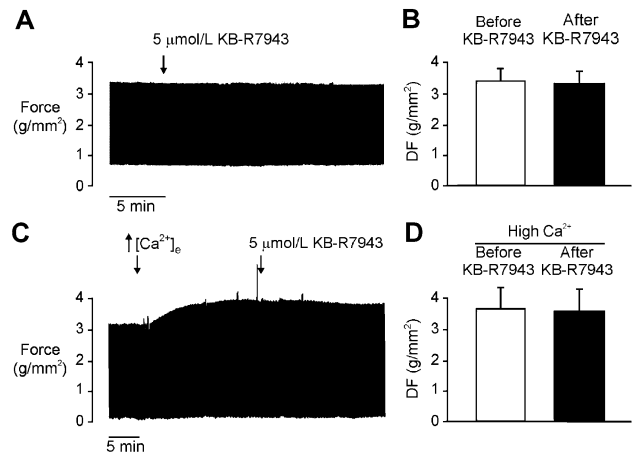


Fig. 8. Inhibition of reverse mode NCX has no effect on basal contractility nor on the increase in contractility promoted by extracellular  $\text{Ca}^{2+}$  increase. Typical force records showing the lack of effect of 5  $\mu\text{mol/l}$  KB-R7943 on basal contractility (A,  $[\text{Ca}^{2+}]_o = 1.35$  mmol/l) and on the increase in contractility of  $\sim 20\%$  promoted by increasing  $[\text{Ca}^{2+}]_o$  from 1.35 to 1.9 mmol/l (C). Overall results of DF (in  $\text{g/mm}^2$ ) for each type of experiments [(B)  $n = 6$  and (D)  $n = 4$ ]. These results strongly suggest that KB-R7943 at this concentration does not exert nonspecific actions that may affect contractility.

promoted by elevating  $[\text{Ca}^{2+}]_o$  from 1.35 to 1.9 mmol/l (panels C and D).

#### 4. Discussion

Several of the effects classically thought to be due to Ang II have been demonstrated to be mediated by the release/formation of ET [1–12]. We are presenting here evidence that the PIE of a low dose of Ang II is mediated by activation of the  $\text{AT}_1$  subtype of receptors and a subsequent release/formation of endogenous ET. Our results, although based only in pharmacological interventions, indicate that Ang II releases endogenous ET that, acting in an autocrine/paracrine fashion, activates the NHE-1, which increases  $[\text{Na}^+]_i$ , activates the reverse mode NCX and promotes  $\text{Ca}^{2+}$  influx to the cell. We should emphasize that our experiments were performed after  $\alpha_1$  and  $\beta_1$  receptors blockade, thus eliminating the contribution of the known Ang II-mediated catecholamine release effect [16].

In the absence of  $\text{pH}_i$  changes, as in our experiments, the PIE of ET (the proposed mediator of Ang II) should very likely due to an increase in the  $\text{Ca}^{2+}$  transient. Yang et al. [28] showed that the increase in the  $\text{Ca}^{2+}$  transient induced by ET-1 is susceptible to inhibition of reverse mode NCX by KB-R7943, and suggested, based on previous work, the possibility of a synergistic contribution of L-type  $\text{Ca}^{2+}$  channels [29,30] and the NCX [28] to the increase in the  $\text{Ca}^{2+}$  transient. Our results are in agreement with this possibility, but we propose that these two effects might be dose dependent and would be dissected along the dose–response curve: while the effect on NCX would be predom-

inant at the beginning of the curve, it will be undetectable at maximal concentrations of ET. We consider that our proposal gives an explanation for a recent report about a lack of NCX contribution to the PIE of maximal doses of Ang II [31], since, under this condition, the increase in  $\text{Ca}^{2+}$  transient through the L-type  $\text{Ca}^{2+}$  channels would be the predominant effect masking the contribution of NCX activity to the increase in  $\text{Ca}^{2+}$  transient. Furthermore, at maximal pCa values, where saturation of  $\text{Ca}^{2+}$  binding sites occurs, a change in the  $\text{Ca}^{2+}$  transient will not affect the “plateau” of the dose–response curve.

Based on previous results from our laboratory [21], we could hypothesize that the Ang II-ET cross-talk is autocrine, but the present data do not allow us to rule out the participation of cells other than myocytes in this mechanism. About the signaling pathway by which Ang II induces the release/formation of ET, we should consider that current evidence suggests that no granules of preformed ET are stored in the cytoplasm [32]; therefore, it should be assumed that ET release depends on de novo peptide formation. In this regard, two mechanisms, not mutually exclusive, might be involved: induction of gene expression [1–5,10] and/or activation of endothelin-converting enzyme [6].

Finally, the question of which ET isoform and ET receptor subtype are involved in the Ang II-ET cross-talk cannot be answered from the present results. Furthermore, the fact that in myocardium, both the ET-1 and ET-3 isoforms and the  $\text{ET}_A$  and  $\text{ET}_B$  subtype of receptors are expressed, makes too speculative any assumption about this matter.

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