Angiotensin II Activates Na^+-Independent Cl^--HCO_3^- Exchange in Ventricular Myocardium

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Abstract—The effect of angiotensin II (Ang II) on the activity of the cardiac Na^+-independent Cl^--HCO_3^- exchanger (anionic exchanger [AE]) was explored in cat papillary muscles. pH_i was measured by epifluorescence with BCECF-AM. Ang II (500 nmol/L) induced a 5-(*N*-ethyl-*N*-isopropyl)amiloride–sensitive increase in pH_i in the absence of external HCO_3^- (HEPES buffer), consistent with its stimulatory action on Na^+-H^+ exchange (NHE). This alkalinizing effect was not detected in the presence of a CO_2-HCO_3^- buffer (pH_i 7.07±0.02 and 7.08±0.02 before and after Ang II, respectively; n=17). Moreover, in Na^+-free HCO_3^-–buffered medium, in which neither NHE nor Na^+-HCO_3^- cotransport are acting, Ang II decreased pH_i, and this effect was canceled by previous treatment with SITS. These findings suggested that the Ang II–induced activation of NHE was masked, in the presence of the physiological buffer, by a HCO_3^--dependent acidifying mechanism, probably the AE. This hypothesis was confirmed on papillary muscles bathed with HCO_3^- buffer that were first exposed to 1 μmol/L S20787, a specific inhibitor of AE activity in cardiac tissue, and then to 500 nmol/L Ang II (n=4). Under this condition, Ang II increased pH_i from 7.05±0.05 to 7.22±0.05 ($P<.05$). The effect of Ang II on AE activity was further explored by measuring the velocity of myocardial pH_i recovery after the imposition of an intracellular alkali load in a HCO_3^--containing solution either with or without Ang II. The rate of myocardial pH_i recovery was doubled in the presence of Ang II, suggesting a stimulatory effect on AE. The enhancement of the activity of this exchanger by Ang II was also detected when the AE activity was reversed by the removal of extracellular Cl^- in a Na^+-free solution. Under this condition, the rate of intracellular alkalinization increased from 0.053±0.016 to 0.108±0.026 pH unit/min (n=6, $P<.05$) in the presence of Ang II. This effect was canceled either by the presence of the AT_1 receptor antagonist, losartan, or by the previous inhibition of protein kinase C with chelerythrine or calphostin C. The above results allow us to conclude that Ang II, in addition to its stimulatory effect on alkaline loading mechanisms, activates the AE in ventricular myocardium and that the latter effect is mediated by a protein kinase C–dependent regulatory pathway linked to the AT_1 receptors. **(*Circ Res*. 1998;82:473-481.)**

Key Words: Na^+-independent Cl^--HCO_3^- exchanger ■ AT_1 receptor ■ angiotensin II ■ myocardial pH_i ■ protein kinase C

Changes in myocardial pH_i can affect a variety of basic cellular functions, including ionic conductances,[1,2] metabolic pathways,[3] Ca^{2+} homeostasis,[4,5] and myofilament sensitivity to Ca^{2+}.[6] In order to ensure strict regulation of pH_i, the mammalian myocardium possesses three well-characterized ion-transport systems. The N^+-H^+ antiporter (NHE) and Na^+-HCO_3^- symport, when activated, induce an intracellular alkalinization,[7–9] whereas the Na^+ independent Cl^--HCO_3^- exchanger (AE) triggers an acidification after an alkaline load.[9–12] Recently, a second acid-loading bicarbonate-independent mechanism has been described.[13] In recent years, many studies have been undertaken on the neurohumoral regulation of each of these transporters. The NHE has been the one most extensively investigated in several tissues including the heart.[7,14–20]

Ang II is known to regulate myocardial contractility[21] and growth.[22] The mobilization of Ca^{2+} and the cardiac hypertrophy occurring under the influence of Ang II stimulation[22–24] have been shown to involve an activation of the phospholipase C, phospholipase D, and possibly phospholipase A_2 pathways.[22,25,26] All of these second messenger systems are activated through the AT_1 receptor.[26] Ang II has been recently shown to increase the activity of two Na^+-dependent proton-extruding mechanisms, the NHE[17] and the Na^+-HCO_3^- symport, in cardiac muscle.[18,19] However, studies on other cells have demonstrated that growth factors (like arginine vasopressin and epidermal growth factor) cause an intracellular acidification in the presence of HCO_3^- because the stimulatory effect on AE activity is greater than the enhancement of the acid-extruding mechanisms.[27,28] In agreement with these observations, we have recently reported that in the presence of the physiological CO_2-HCO_3^- buffer, the positive inotropic effect of Ang II occurs in the absence of any change in pH_i.[29] The lack of change in myocardial pH_i after Ang II in muscles that were

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Selected Abbreviations and Acronyms

AE = anionic exchange(r)
Ang II = angiotensin II
CHE = chelerythrine chloride
EIPA = 5-(*N*-ethyl-*N*-isopropyl)amiloride
NHE = Na^+-H^+ exchange(r)
NMDG = *N*-methyl-D-glucamine
PKC = protein kinase C
TMA = trimethylamine hydrochloride

exposed to a bicarbonate-containing solution suggested to us that a stimulatory effect on a bicarbonate-dependent acid-loading mechanism was superimposed on the known effect of Ang II on NHE. Therefore, the aim of the present study was to focus on the effect of Ang II on the AE activity in particular and to characterize the nature of the membrane receptor and intracellular signaling pathway through which such an interaction might be mediated.

Materials and Methods

The experiments were performed in thin cat papillary muscles (mean cross-sectional area, 0.47 ± 0.03 mm^2; n=58) dissected from the right ventricle. The animals were anesthetized with pentobarbital sodium (35 mg/kg IP), and the hearts were quickly removed and placed in standard HCO_3^--buffered solution (see below). The general procedure for dissecting and mounting the muscles has been already described.[30] After they were mounted, the muscles were progressively stretched to reach the length at which maximal developed force was obtained. Muscles were paced with square-wave pulses of 10-millisecond duration, and a voltage intensity 10% over threshold was applied via two platinum wires running along the sides of the preparations. Mean developed force of isometric contractions (at the end of the washout period that followed the loading process with the fluorescent probe) was 0.73 ± 0.13 g/mm^2. Experiments were performed at 30°C, and the muscles were superfused at a constant flow of 4.0 mL/min with one of the following solutions: (1) The HEPES-buffered solution contained (mmol/L) NaCl 146.2, KCl 4.5, $CaCl_2$ 1.35, $MgSO_4$ 1.05, glucose 11.0, and HEPES 5.0. pH was adjusted to 7.4 at 30°C with 3 mol/L NaOH (total [Na^+], 148.5 mmol/L) and then gassed with 100% O_2. (2) HCO_3^--buffered solutions were equilibrated with a CO_2-O_2 gas mixture that gave a pH value of 7.39 ± 0.01. The standard HCO_3^--buffered solution contained (mmol/L) NaCl 128.3, KCl 4.5, $CaCl_2$ 1.35, $NaHCO_3$ 20.23, $MgSO_4$ 1.05, and glucose 11.0. For Na^+-free HCO_3^-–buffered solution, NaCl and $NaHCO_3$ were replaced with equimolar amounts of choline chloride and NMDG, respectively. NMDG · HCO_3 was then generated in situ by prolonged (≈2-hour) bubbling of the solution with a gas mixture of CO_2 in O_2 to give a final pH value of 7.40. Cl^--free, Na^+-free, low-Ca,$^{2+}$ HCO_3^--buffered solution contained (mmol/L) NMDG 148.0, $MgSO_4$ 1.05, K_2SO_4 2.25, $CaCO_3$ 0.10, aspartic acid 135.0, and glucose 11.0. All the experiments were performed in the presence of 1.0 μmol/L atenolol (Sigma Chemical Co) plus 1.0 μmol/L prazosin (Sigma) in order to prevent adrenoceptor activation by the possible release of catecholamines from nerve endings. Ang II (Sigma) was added to the superfusate in appropriate amounts to give a final concentration of 500 nmol/L. NHE activity was inhibited with EIPA (Research Biochemicals Intl) at 5 μmol/L, whereas SITS (Sigma) was used at 0.1 mmol/L to inhibit AE mechanisms. SITS was dissolved in the superfusate immediately before use, and the solution was protected from light to prevent photodegradation of the drug. CHE (Research Biochemicals Intl) and calphostin C (Research Biochemicals Intl) were used at 10 μmol/L and 50 nmol/L, respectively, as specific inhibitors of PKC.[31,32] Losartan, which was used at 10 μmol/L to block the AT_1 receptor subtype, was a generous gift of Peter K.L. Siegl from Merck Sharp & Dohme, West Point, Pa. The specific inhibitor of AE activity, S20787,[33] was used at 1 μmol/L and was a kind donation of Elizabeth Scalbert from the Institut de Recherches Internationales Servier, Courbevoie, France.

pH_i Measurements.

Measurements of pH_i in the isolated muscles were made after loading the muscles with the acetoxymethyl ester form of the pH-sensitive dye BCECF (BCECF-AM, Molecular Probes) as previously described.[30] BCECF fluorescence was excited at 450 and 495 nm, and the fluorescence emission was monitored after passage through a 535 ± 5-nm filter. To limit photobleaching, a neutral-density filter (1% transmittance) was placed in the excitation light path, and a manual shutter was used to select sampling intervals (for 3 seconds every 15 seconds) during the protocol. At the end of each experiment, fluorescence emission was calibrated by the high K^+–nigericin method.[34] The calibration solution contained (mmol/L) KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 5.0, nigericin 0.01, sodium cyanide 4.0, and 2,3-butanedione monoxime 20.0 (the last to prevent muscle contracture[35]). Buffer pH was adjusted with KOH to four different values ranging from 7.5 to 6.5. Such a calibration gave a linear relation ($r=.99\pm.003$, n=58) between buffer pH values and the fluorescence ratio (F_{495}/F_{450}), with the latter calculated as follows:

F_{495}/F_{450}=(fluorescence at 495 nm−autofluorescence at 495 nm)/(fluorescence at 450 nm−autofluorescence at 450 nm)

The experimental protocols designed for the present study were as follows: basal myocardial pH_i was noted after 15 minutes of superfusion with HEPES- or the other HCO_3^--buffered solutions indicated above. The muscles were then exposed to Ang II for a further 30 minutes. To study the effect of Ang II on the activity of the AE in its "forward" and "reverse" modes, the muscles were subjected either to intracellular alkaline loads[11,13,36] or to the removal of extracellular chloride,[16,37,38] respectively.

Intracellular alkalinization was induced by exposing the tissue to TMA (20.0 mmol/L), a technique that has been already validated for the study of the activity of the AE.[11,36] The preparations were subjected to two consecutive transient exposures to TMA. After the first alkaline pulse, the muscles were superfused with standard HCO_3^--buffered solution to allow further recovery of pH_i before the second exposure to TMA (second alkaline pulse). As previously reported by several investigators, the initial rate of pH_i recovery (dpH_i/dt_i, in pH unit/min) was estimated from the slope of the straight lines fitted to the values of pH_i recorded during the first 3 minutes after the peak of alkalosis by linear least squares regression analysis.[11,13,36] The correlation coefficient (r) values of the linear fits ranged from .78 to .96. In control experiments, both TMA pulses occurred under identical conditions, and no significant difference in the rate of pH_i recovery was detected. When studying the effect of Ang II, the peptide was included in the superfusate 30 minutes before the second pulse to the end of the experiment. Intracellular buffering power (β) was calculated during the alkaline and acid load of the TMA pulses, in the absence and presence of Ang II, using the following equation:

$$\beta \text{ (in mmol/L)} = \Delta[TMAH^+]_i/\Delta pH_i$$

where ΔpH_i is the change in pH_i measured immediately after the addition or withdrawal of TMA and $\Delta[TMAH^+]_i$ was calculated on the basis of the rearrangement of the Henderson-Hasselbach equation:

$$[TMAH^+]_i=[TMA]_o\times10^{(pH_o-pH_i)}/1+10^{(pH_o-pK)}$$

with the dissociation constant (pK) taken as 9.8. Although the differences did not reach statistical significance, an ≈20% increase in β was detected in the presence of Ang II at each pH_i analyzed. At a mean pH_i of 7.16 ± 0.03, β values were 58.2 ± 5.9 and 73.2 ± 7.6 mmol/L (n=6, P=NS by paired t test) in the absence and in the presence of Ang II, respectively, whereas values of 53.6 ± 20 and 64.6 ± 16 were determined at 7.43 ± 0.04 (n=6, P=NS by paired t test).

After chloride removal from the extracellular space, the activity of the AE operating in the "reverse" mode was characterized from the initial rate of the increase in pH_i immediately after Cl^- deprivation. The activity of the Na^+-independent AE can be assessed specifically once Na^+ is omitted from the Cl^--free superfusate. Nevertheless, in

order to minimize possible changes in pH_i resulting from the entry of either Ca^{2+} or H^+ as the result of the reversal of the Na^+-Ca^{2+} or Na^+-H^+ exchangers, respectively, these experiments were carried out at low [Ca^{2+}] and in the presence of EIPA. The values of pH_i determined during the removal of Cl^- were fitted to a straight line (by linear least squares fit of the pH_i values recorded during the first 3 minutes[37]) and also to an exponential curve of the form $\Delta pH_{it}=\Delta pH_{i\infty}(1-e^{-k\Delta t})$, where ΔpH_{it} and $\Delta pH_{i\infty}$ are the changes in pH_i from the initial value at time t and after steady state has been reached, respectively, and k is the rate constant. Even though the r values obtained from the fitting to the exponential curve were higher (r=.86 to .99) than those obtained by linear regression (r=.61 to .98), the initial rate of the changes in pH_i (dpH_i/dt_i in pH unit/min) was determined by both procedures. Although a biexponential probably fit better than a monoexponential for the entire curve, the initial rate of the changes in pH_i was essentially the same when estimated either with monoexponential or biexponential fits. Mean percent difference between both estimates was 1.25±1.58 (n=28). Two consecutive Cl^--removal protocols were carried on the same muscle preparation, the first in the absence and the second in the presence of the specified compounds. The values of dpH_i/dt_i obtained during the first and second protocol were then compared. Previous experiments have shown that dpH_i/dt_i estimated in two successive Cl^--removal protocols, under control conditions, did not differ from each other. When the effects of AT_1 receptor subtype blockade or PKC activity inhibition were studied, losartan, CHE, or calphostin C were, accordingly, added to superfusate 10 minutes before Ang II. In the experiments with calphostin C, only one Cl^--removal protocol was performed, and the results obtained were compared with those in the absence of Ang II, pooling the overall protocols (n=20) under this condition. Neither losartan nor PKC inhibitors produced significant changes in resting pH_i.

Statistics

Data are expressed as mean±SEM. Statistical analysis of data was performed by paired t test and ANOVA followed by Bonferroni's test, as appropriate. Probability of null hypothesis <5% (P<.05) was considered significant.

Results

Action of Ang II on Resting Myocardial pH_i

The effect on pH_i of various concentrations of Ang II ranging from 10 nmol/L to 1 μmol/L was tested on pilot experiments carried out in HEPES-buffered medium. The magnitude of the effect for a 30-minute exposure to Ang II was related to its concentration in the superfusate. The Ang II–induced rise in pH_i was maximal at a concentration of 1 μmol/L; 30% and 96% of the maximal effect was obtained with 10 and 500 nmol/L, respectively. In accordance with data reported by other investigators,[19,21,39] the maximal inotropic effect was obtained with 500 nmol/L Ang II. Although this concentration seems to be higher than reported values in extracellular fluids,[25,40] it was chosen for all the experiments because it was, in the absence of bicarbonate, eliciting the maximal contractile response and nearly the maximal increase in pH_i.

The steady-state pH_i is determined by the balance between processes that load cells with acid equivalents and those that extrude them. In the absence of a CO_2-HCO_3^- buffer system, the only substantial acid-extruding mechanism is NHE. Fig 1A illustrates an experiment in which a papillary muscle, superfused with a nominal HCO_3^--free (HEPES) buffer, was exposed to 500 nmol/L Ang II. As shown in Fig 1A, pH_i began to increase after 12 minutes of exposure to the peptide, reaching a value 0.11 U more alkaline than the initial one after 30 minutes. Fig 1B shows mean values of basal pH_i and those after 30 minutes of exposure to Ang II in HEPES-buffered medium (n=4).

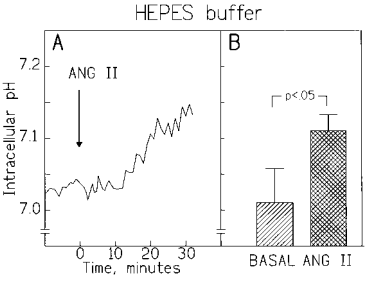

Figure 1. Effect of Ang II on myocardial pH_i in nominally HCO_3^--free (HEPES) buffer. A, The data are from a representative experiment showing the time course of the effect of Ang II on resting pH_i. B, The mean±SEM values obtained from all the experiments (n=4) both before (basal) and after 30 minutes of exposure to Ang II at 500 nmol/L are shown. The significance of the difference between the basal and Ang II–stimulated pH_i values was assessed by paired t test.

The results shown in Fig 2 confirm that the increase in pH_i elicited by Ang II in the absence of HCO_3^- resulted from the stimulation of NHE. The blockade of NHE activity by 5 μmol/L EIPA prevented the increase in pH_i induced by Ang II (n=6). Indeed, when the NHE was inhibited in nominal HCO_3^--free solution, a slight but significant decrease in pH_i was detected. A similar decline in pH_i after exposure to Ang II in the absence of NHE activity was previously observed by several authors and was attributed to a metabolic effect of Ang II.[17,41]

With HCO_3^- present in the superfusate, however, the same dose of Ang II was followed by no significant changes in myocardial pH_i (Fig 3A). Here, the fact that no significant increase in pH_i could be detected despite the stimulatory effect of Ang II on the NHE suggested the possibility of a simultaneous enhancement of a bicarbonate-dependent acidifying mechanism. The possibility that Ang II depressed the activity of the Na^+-HCO_3^- symport and blunted the increase in pH_i is unlikely, since the cotransporter has been shown to be stimulated by the peptide.[18,19]

Fig 4 shows the effect of Ang II when examined in HCO_3^- buffer after the blockade of NHE activity with EIPA. A slight

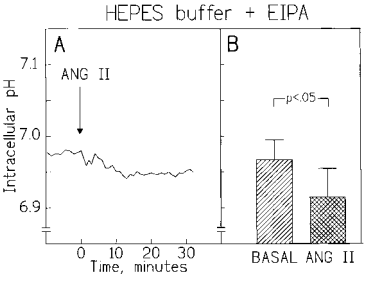

Figure 2. Prevention of the alkalinizing effect of Ang II in HEPES buffer by blockade of NHE. A, The data are from a representative experiment showing the suppression of the alkalinizing effect of Ang II in the presence of the specific NHE inhibitor, EIPA. Here, instead of the increase in pH_i observed in Fig 1, a slight acidification was induced by Ang II under these conditions. B, The mean±SEM values obtained from all the experiments (n=6) both before (basal) and after 30 minutes of exposure to Ang II at 500 nmol/L in the presence of 5 μmol/L EIPA are shown. The significance of the difference between the basal and Ang II–stimulated pH_i values was assessed by paired t test.

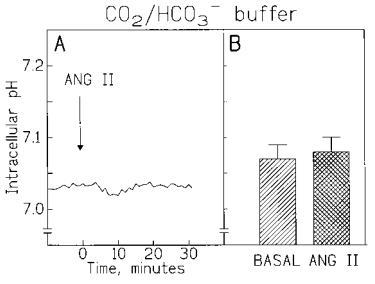

Figure 3. Absence of alkalinizing effect of Ang II in the presence of the physiological CO_2-HCO_3^- buffer. A, The data show the time course of the effect of Ang II on pH_i in a representative experiment performed on a papillary muscle superfused with CO_2-HCO_3^- buffer. B, The mean±SEM values obtained from all the experiments (n=17) before (basal) and after 30 minutes of exposure to Ang II at 500 nmol/L are shown.

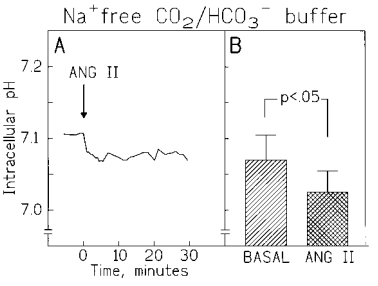

Figure 5. Effect of Ang II on myocardial pH_i in Na^+-free HCO_3^- buffer. A, The data are from a representative experiment showing an acidifying effect of Ang II in the absence of external Na^+ ions. B, The mean±SEM values obtained from all the experiments (n=5) both before (basal) and after 30 minutes of exposure to Ang II at 500 nmol/L are shown. The significance of the difference between the basal and Ang II–stimulated pH_i values was assessed by paired *t* test.

transitory decrease in pH_i followed by a return to baseline was detected in each of the seven experiments. Although these results are difficult to interpret, a reasonable hypothesis would be that Ang II stimulated AE activity (thereby decreasing pH_i), which was then followed by a recovery mediated through activation of Na^+-HCO_3^- cotransport. The stimulation of symport activity could have been triggered by the fall in pH_i and/or a stimulatory effect of Ang II on this mechanism.[18,19]

The experiments shown in Fig 5 give further support to the hypothesis of a stimulatory effect of Ang II on AE activity. In this series of experiments, the effect of Ang II in Na^+-free HCO_3^-–buffered medium was examined. In order to minimize the progressive decrease in pH_i that could result from background acid loading in the absence of external Na^+, the muscles were exposed to Na^+-free solutions only for a short period (≈15 minutes). Under this condition, in which neither NHE nor the symport was operative, the application of Ang II resulted in a clear decrease in pH_i, indicating that the peptide stimulated a Na^+-independent HCO_3^--dependent acidifying mechanism (Fig 5). Moreover, this acidifying effect of Ang II in Na^+-free HCO_3^-–buffered solution was abolished by pretreatment of the preparations with SITS (average ΔpH_i being 0.008±0.02, n=4). Although the magnitude of the decrease in pH_i was similar to the one obtained when NHE activity was blocked in HEPES buffer, it should be considered that in the presence of HCO_3^-, the total buffer capacity is increased, attenuating any change in pH_i for a given acid equivalent flux.

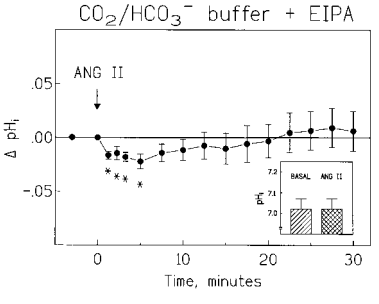

Figure 4. Effect of Ang II on myocardial pH_i in HCO_3^- buffer after the inhibition of NHE activity. A slight transitory decrease in pH_i followed by a return to baseline value was observed after the application of 500 nmol/L Ang II to muscles superfused with HCO_3^- buffer containing 5 μmol/L EIPA (n=7). Inset shows values of pH_i before (basal) and after 30 minutes of exposure to Ang II. *P<.05 vs baseline.

The previous results show that in the presence of extracellular HCO_3^-, Ang II stimulation of NHE activity was counteracted by an acidifying mechanism that was SITS sensitive and independent of external Na^+. This bicarbonate-dependent acid loading was probably mediated by AE activity. Perhaps the strongest evidence of the stimulatory effect of Ang II on the AE is apparent in the results shown in Fig 6. In these experiments, the effect of Ang II in HCO_3^--buffered medium was assessed in the presence of S20787, a recently described specific inhibitor of AE activity.[33] Under this condition, an increase in pH_i was detected after application of Ang II. The blockade of AE activity seemed, therefore, to unmask an Ang II–induced stimulatory effect on the alkaline loaders and resulted in clearly apparent intracellular alkalinization. The effectiveness of S20787 as an inhibitor of AE activity was tested in parallel experiments that proved that S20787 at 1 μmol/L reduced by 67±12% the rate of pH_i recovery from an intracellular alkaline load without altering steady-state pH_i value (n=3, data not shown). These results are in agreement with those previously reported by Lagadic-Gossmann et al.[33]

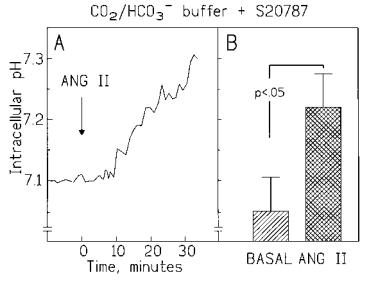

Figure 6. Ang II increases pH_i in CO_2/HCO_3^- buffer in the presence of a specific inhibitor of AE activity. A, The data are from a representative experiment showing the alkalinizing effect of Ang II in the presence of S20787, a specific inhibitor of AE activity. B, The mean±SEM values obtained from all the experiments (n=4) both before (basal) and after 30 minutes of exposure to Ang II at 500 nmol/L are shown. The significance of the difference between the basal and Ang II–stimulated pH_i values was assessed by paired *t* test. At the concentration used (1 μmol/L), S20787 did not change the steady-state pH_i. In accordance with previously reported data,[32] this concentration of S20787 inhibited by ≈70% the activity of AE as measured by the rate of recovery from intracellular alkalinization in control experiments (n=3).

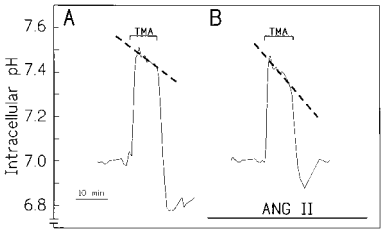

Figure 7. Effect of Ang II on the recovery of pH_i after an intracellular alkaline load. The data are from a representative experiment among the six performed. Intracellular alkalosis was imposed by rapid addition of TMA (20.0 mmol/L) to HCO_3^--buffered superfusate. A, The sudden TMA-induced elevation in pH_i is followed by a gradual recovery of pH_i. The rate of the pH_i recovery was estimated by the slope of the line fitted by least squares method to the values of pH_i after the peak of TMA-induced alkalosis (broken line). B, The same protocol as in panel A was used, except that 500 nmol/L Ang II was applied 30 minutes before the TMA pulse. In the presence of Ang II, a marked increment in the rate of pH_i recovery from alkalosis was detected. In control experiments, we showed that the rates of pH_i recovery were the same in two consecutive TMA pulses (see "Materials and Methods").

To further explore the effect of Ang II on AE activity, the following experiments, in which the exchanger was activated while operating in its "forward" and "reverse" modes, were designed.

Action of Ang II on the Activity of the AE After an Intracellular Alkaline Load

During intracellular alkalosis, a recovery of pH_i resulting from an extrusion of intracellular HCO_3^- ions in exchange for extracellular Cl^- can be detected, regardless of the technique used to increase pH_i.[10–12] Exposure to TMA has been previously demonstrated to be a valid technique for investigating the activity of the AE because no recovery from TMA-induced intracellular alkalosis is detected in HCO_3^--free solutions.[11,42] Papillary muscles were exposed to TMA, and the velocity of the recovery of pH_i was measured before and after Ang II as an indication of the activity of the AE. Fig 7 shows the results of one of six similar experiments. Under control conditions (Fig 7A), pH_i rose rapidly from a steady-state value of 7.00 to 7.51 after exposing the tissue to TMA. The intracellular alkalosis was followed by a recovery in pH_i due to the activity of the AE. The rate of pH_i recovery from the intracellular alkaline load was enhanced in the presence of Ang II (Fig 7B). On average, in these experiments, exposure to TMA increased pH_i from 7.12±0.04 to 7.49±0.06, and the average rate of pH_i recovery was 0.009±0.003 pH unit/min in the absence of Ang II (n=6). In the presence of Ang II, TMA caused an elevation in pH_i from 7.12±0.03 to 7.46±0.05, but even though the alkaline load was of a magnitude similar to that under control conditions, the velocity of pH_i recovery was increased to 0.018±0.004 pH unit/min ($P<.05$ by paired t test), suggesting an enhanced HCO_3^- efflux. However, it should be also considered that the higher rate of recovery could be merely the result of a decrease in buffering power induced by Ang II. Buffer capacity was estimated as explained in "Materials and Methods," and although ≈20% of increase in β was detected in the presence of Ang II, the difference did not reach statistical

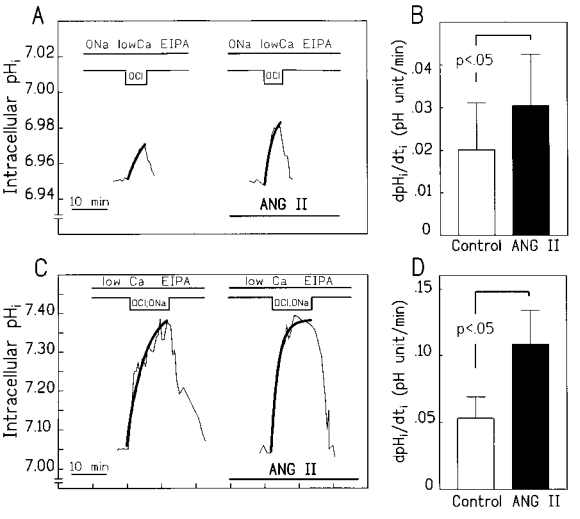

Figure 8. Stimulation by Ang II of the activity of AE operating in "reverse mode." The activity of the anion exchanger was reversed into a mode of intracellular Cl^-–extracellular HCO_3^- exchange by exposure of the myocardium to Cl^--free superfusate. The superfusate was also made Na^+ free to ensure the Na^+ independence of the exchange activity being assayed, and it also contained low Ca^{2+} (100 μmol/L) and EIPA (5 μmol/L) to eliminate the possible influence of Na^+-H^+ and Na^+-Ca^{2+} exchanger reversal in a Na^+-free milieu. A, Results from a representative experiment in which the preparation was superfused with Na^+-free medium for ≈60 minutes before the first Cl^- removal (left, no Ang II in the medium). The Cl^- removal protocol was repeated after 30 minutes of exposure to 500 nmol/L Ang II (right). Experimental data were fitted to an exponential curve of the form $\Delta pH_{it}=\Delta pH_{i\infty}(1-e^{-k\Delta t})$ (where ΔpH_{it} and $\Delta pH_{i\infty}$ are the changes in pH_i from the initial value at time t and after steady state has been reached, respectively, and k is the rate constant) to estimate the initial velocity of the rise in pH_i (dpH_i/dt_i in pH unit/min) as explained in "Materials and Methods." The theoretical curves corresponding to each set of data are shown; the correlation coefficient (r) values were .93 and .98, without and with Ang II, respectively. B, Mean dpH_i/dt_i value±SEM obtained from the overall experiments (n=4) in Na^+-deprived medium. C, Results from a representative experiment in which Na^+ withdrawal was effected simultaneously to Cl^- removal, in the absence (left) and after exposing the muscle to Ang II for 30 minutes (right). The theoretical curves obtained by exponential fit of each set of data are shown, and their corresponding r values were .92 and .99 without and with Ang II. D, Mean values of initial rate of alkalinization induced by Cl^- removal in the experiments under the conditions indicated in panel C (n=6). The significance of the differences between values of dpH_i/dt_i values before and after Ang II was assessed by the paired t test. Note that Ang II significantly increased the rate of intracellular alkalinization, regardless of whether Na^+ deprivation was before or simultaneous with Cl^- withdrawal.

significance. Therefore, changes in dpH_i/dt_i were considered to be proportional to changes in bicarbonate fluxes.

Action of Ang II on the Activity of the AE Operating in the Reverse Mode

When myocardial tissue is suddenly subjected to the removal of extracellular Cl^-, pH_i rises because of the influx of HCO_3^- resulting from the reversal of AE activity. To measure specifically the activity of this AE, the removal of Cl^- was performed in Na^+-free solutions. Moreover, the $[Ca^{2+}]$ in this solution had been lowered to prevent possible pH_i shifts resulting from changes in $[Ca^{2+}]_i$, and the blockade of NHE was also achieved using EIPA so as to prevent a possible reversal of the anti-

porter.[11,43] Under Cl^--free conditions, the activity of the AE could then be estimated from the initial velocity of alkalinization (dpH_i/dt_i) as used before by other investigators.[37,38,43]

Fig 8A shows the changes in pH_i obtained in a typical experiment after Cl^- removal with a muscle kept in Na^+-free medium for a period longer than an hour, either in the absence (left) or in the presence of Ang II (right). Under these experimental conditions, the removal of extracellular Cl^- caused an alkalinization that was readily reversed on readdition of Cl^-. Since extracellular Na^+ was absent in these experiments, the mechanism that produced this alkalinization must have been Na^+ independent. The theoretical curves obtained by the exponential fits are shown superimposed to the pH_i tracings in Fig 8A. Fig 8B shows the initial rates of intracellular alkalinization induced by Cl^- removal estimated by these fittings (initial pH_i values were 6.94 ± 0.04 and 6.92 ± 0.03 in the absence and presence of Ang II, respectively; n=4). Ang II accelerated the initial rate of alkalinization induced by Cl^- removal in the absence of Na^+. The acceleration induced by Ang II in the rate of alkalinization was also noted when it was determined by the fitting of the pH_i values recorded after Cl^- removal to a straight line (instead of the exponential fitting). In this case, the rate of Cl^- removal–induced alkalinization increased from a control value of 0.011 ± 0.007 to 0.019 ± 0.002 pH unit/min under Ang II ($P<.05$ by paired t test).

Fig 8C shows the results from a representative experiment in which Na^+ and Cl^- were simultaneously removed in order to minimize the induction of intracellular acidosis and a consequent inactivation of AE.[10,38,44] The theoretical curves obtained by the exponential fits are, like in Fig 8A, shown superimposed to the pH_i tracings. The average values of the initial rate of alkalinization estimated in the experiments of this group are illustrated in Fig 8D. Ang II increased the initial rate of alkalinization induced by the removal of external Cl^- (baseline pH_i was 7.07 ± 0.04 and 7.08 ± 0.04 in the absence and presence of Ang II, respectively; n=6). The effect of Ang II was also detected when the initial rate of pH_i change was estimated by fitting the data to a straight line. Mean value was 0.045 ± 0.01 pH unit/min in the absence of Ang II, and it increased to 0.077 ± 0.02 pH unit/min in the presence of Ang II (n=6, $P<.05$ by paired t test).

Ang II was, then, showing a stimulatory effect on the AE working in reverse mode under rigorous Na^+-free conditions and also when Na^+ and Cl^- deprivation were simultaneously performed. The initial rate of alkalinization, the extent of alkalinization, and the magnitude of the changes induced by Ang II after prolonged Na^+ deprivation were, however, only about half of those observed when Na^+ deprivation was simultaneous with Cl^- withdrawal. This raises the question of whether the increased activity and responsiveness of the AE under less strict Na^+ deprivation is due to (1) the presence of residual Na^+ stimulating a Na^+-dependent Cl^--HCO_3^- exchange or (2) the higher pH_i, which stimulates AE activity. This question was addressed by Boyarsky et al[38] who showed that in renal mesangial cells the increase in pH_i following the removal of Cl^- is mediated by the AE, regardless of whether the experiments were carried out in the presence or absence of Na^+.[38] The authors demonstrated that the reduction in intracellular alkalinization evoked by Cl^- removal after Na^+ deprivation was only reflecting the inhibition of AE activity by low pH_i, as previously reported.[10,44] If, in addition, we consider that a Na^+-dependent Cl^--HCO_3^- exchange has not been demonstrated in adult cardiac myocytes,[8] it is very likely that Na^+ deprivation is not necessary to assess AE activity after Cl^- removal.

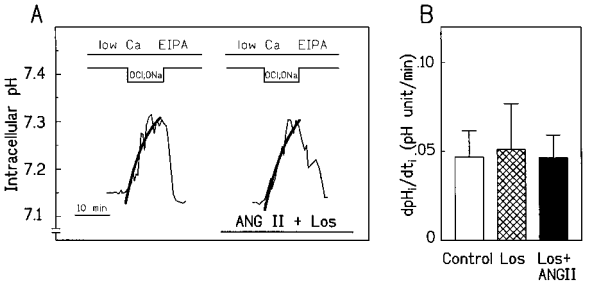

Figure 9. Involvement of the AT_1 receptor subtype in the stimulation of AE by Ang II. A, Experimental conditions are the same as those in Fig 8C except that losartan (Los), a specific antagonist of AT_1 receptor subtype, was added (10 μmol/L) 10 minutes before Ang II. The pH_i values obtained in a representative experiment with the same papillary muscle before (left) and after exposure to Ang II+Los (right) are depicted as a function of time. The theoretical exponential curves fitted to the experimental data are shown; the r values were .86 and .88 in the absence and presence of Ang II, respectively. B, Mean dpH_i/dt_i values were obtained in the control condition (no drugs present, n=8), in the presence of 10 μmol/L Los (n=4), and in the presence of Los+Ang II (n=4). No significant difference in the rate of intracellular alkalinization induced by Cl^- removal was detected when Los or Los+Ang II was added (ANOVA).

After demonstrating that the activation of AE activity by Ang II could be detected by Cl^- deprivation in the absence of Na^+ and when Na^+ deprivation was simultaneous with the removal of Cl^-, we used the same protocol as shown in Fig 8C to assess the subtype of AT receptor involved and the possible role played by PKC activity. Fig 9A shows one of four similar experiments in which losartan (10 μmol/L) was included in the superfusate before testing the effect of Ang II. The Ang II–induced increase in the rate of intracellular alkalinization following Cl^- removal was eliminated when the AT_1 receptors were blocked. The mean values of dpH_i/dt_i under basal conditions (no drugs) and in the presence of losartan and Ang II plus losartan are shown in Fig 9B. Blockade of AT_1 receptors with losartan did not change the rate of intracellular alkalinization induced by Cl^- removal and canceled the acceleration induced by Ang II (Fig 9B). The suppression by losartan of the effects of Ang II was also detected when dpH_i/dt_i values were determined by linear regression (0.045 ± 0.02 versus 0.044 ± 0.01 pH unit/min, n=4).

In order to gain further insight into the mechanism by which Ang II enhances the activity of the AE, experiments similar to those described in Figs 8C and 9A were performed but in the presence of the selective PKC inhibitor, CHE. Fig 10A presents the results of a representative experiment in which, like the preceding experiments, the preparations were subjected to Cl^- deprivation first in the absence of Ang II (left) and then in the presence of Ang II and CHE (right). Fig 10B shows the initial rate of changes in pH_i induced by Cl^- removal under control conditions and in the presence of CHE or Ang

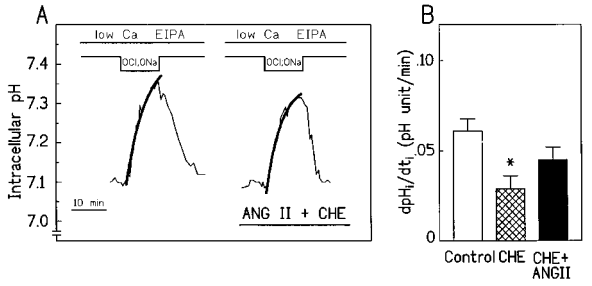

Figure 10. Abolition of Ang II–induced stimulation of AE through inhibition of PKC activity. Experimental conditions are the same as those stated in Figs 8C and 9A except that the specific inhibitor of PKC activity, CHE (10 μmol/L), was added 10 minutes before Ang II. A, The pH_i values obtained with the same papillary muscle before (left) and after exposure to Ang II+CHE (right) are depicted as a function of time. The theoretical exponential curves fitted to the experimental data are shown, and the *r* values were .98 for both curves. B, Mean dpH_i/dt_i values were obtained under control conditions (no drugs added, n=8), in the presence of CHE (n=4), and in the presence of CHE+Ang II (n=4). Inhibition of PKC activity decreased the velocity of intracellular alkalinization after Cl^- removal and canceled the acceleration induced by Ang II. **P*<.05 vs control (ANOVA).

II plus CHE. PKC inhibition by CHE decreased the initial rate of alkalinization. Application of Ang II in the presence of CHE did not significantly change the initial rate of alkalinization (Fig 10B) compared with the control value or with CHE without Ang II (ANOVA). Then, the acceleration in the initial rate of alkalinization seen with Ang II alone was no longer detected in the presence of the PKC inhibitor, either when estimated by linear (0.0395±0.007 versus 0.0330±0.008 pH unit/min, n=4) or exponential fittings (Fig 10B). A similar suppression of the effects of Ang II on the activity of the AE was also obtained when another structurally and mechanistically different inhibitor of PKC activity, calphostin C, was used. Under this condition, the initial rate of alkalinization estimated with the exponential fit was 0.0344±0.007 pH unit/min and with the linear fit was 0.017±0.005 pH unit/min (n=4, *P*<.05 compared with Ang II and not significantly different from control by ANOVA).

Discussion

An increase in pH_i resulting from the activation of NHE by Ang II was observed in the absence of bicarbonate. However, when bicarbonate was present, we were unable to detect any significant change in pH_i after Ang II. The lack of any detectable alkalinization in the presence of bicarbonate, being NHE operative, suggested that the activation of AE by Ang II was a strong possibility. The following pieces of evidence gave further support to this hypothesis: (1) when AE activity was specifically inhibited, Ang II induced a rise of myocardial pH_i in the presence of bicarbonate; (2) when in the presence of bicarbonate, the alkaline loaders (NHE and Na^+-HCO_3^- symport) were rendered quiescent by Na^+ deprivation, Ang II induced a decrease in myocardial pH_i that reflected its stimulatory effect on AE activity; (3) when AE activity was challenged by an intracellular alkaline load (a maneuver validated to assess the activity of this exchanger), Ang II enhanced the velocity of pH_i recovery; and (4) Ang II increased the rate of intracellular alkalinization due to the reversal of AE activity that follows the removal of Cl^- from bicarbonate-buffered solutions.

Ang II can bind to at least two different types of receptors, referred to as AT_1 and AT_2.[45] The ability of losartan to block the stimulation of the AE would indicate that the effect of Ang II on the AE is mediated by the AT_1 receptor subtype. This finding is consistent with recent reports indicating that most of the effects of Ang II on heart muscle involve the participation of AT_1 receptors.[19,21,22,25,26] Nevertheless, the action of Ang II on Na^+-HCO_3^- cotransport in cultured neonatal rat ventricular myocytes has been found to occur through binding to receptors of the AT_2 subtype.[18] However, it is important to emphasize that the AT_2 receptor subtype is only transiently expressed in the neonatal rat heart.[46]

AT_1 receptors belong to the class of seven transmembrane domain receptors coupled to G regulatory proteins, and their activation by Ang II involves a number of intracellular second messengers. Ang II stimulates phospholipase C, resulting in a subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate and the formation of inositol 1,4,5-trisphosphate and diacylglycerol. Ang II has also been shown to activate the mitogen-activated protein kinase pathway.[47] Therefore, Ang II, like other humoral stimuli known to act through receptors coupled to G_q regulatory proteins, stimulates multiple intracellular phosphotransferase reactions mediated by both PKC-dependent and PKC-independent pathways.[24,47] Furthermore, evidence had been presented showing that the NH_2-terminal domain of the AE_3 protein expressed in brain and heart plays a regulatory role in the activity of the AE,[48] and Yannoukakos et al[49] noted that the AE_3 isoform of cardiac muscle contains several potential PKC consensus phosphorylation sites. Ludt et al[50] reported that in Vero cells downregulation of PKC was followed by a change in the sensitivity of the AE to intracellular proton concentration. Our observation that the inhibition of PKC canceled the stimulatory effect of Ang II on the AE activity would further suggest that its action very likely involves a PKC-dependent phosphorylation of the exchanger-protein molecule itself.

Studies on other cells have demonstrated that growth factors (eg, arginine vasopressin and epidermal growth factor) raise pH_i by stimulating the NHE. However, in the presence of bicarbonate, growth factors cause an acidification because the stimulatory effect on AE activity is greater than the enhancement of the acid-extrusion mechanisms.[27,28] We now show that another growth factor, Ang II, activates the AE of the heart, operating in either the forward or the reverse mode. During the course of the present experiments on cat myocardium, we were aware, by a preliminary communication, that Ang II was shown to have a stimulatory effect on the activity of AE in the perfused ferret heart.[51] However, a more recent study by the same authors reported a lack of action of Ang II on the activity of the AE in the same preparation.[19]

The AE, in addition to mediating the Cl^- "shift" in erythrocytes,[52] operates as an alkali extruder that regulates pH_i in diverse tissues, including lymphocytes,[53] smooth muscle cells,[54] and myocardium.[10,11,16] In cardiac muscle, the AE may be an important mechanism for maintaining $[Cl^-]_i$ above the level otherwise obtained at electrochemical equilibrium.[49,55] Cl^- channels, which may also contribute to the regulation of

$[Cl^-]_i$, have been shown to be activated by Ang II in cardiomyocytes.[56]

We conclude that Ang II enhances the activity of the AE in ventricular myocardium and that this effect is mediated by the AT_1 receptor subtype and involves a PKC-dependent regulatory pathway. The fact that in the presence of bicarbonate no changes in myocardial pH_i were detected in our experiments does not argue against an increase in $[Na^+]_i$ due to stimulation of NHE in the presence of Ang II. The increase in $[Na^+]_i$ may still be present even if the effects of the increased activity of NHE on pH_i were blunted by the enhanced activity of the AE. This $[Na^+]_i$ increase will lead to a secondary increase in $[Ca^{2+}]_i$ through the Na^+-Ca^{2+} exchanger mechanism, and this may contribute to the positive inotropic effect of Ang II. However, the effect of Ang II on the amplitude of Ca^{2+} transients is controversial,[41,57,58] and a synergistic action of $[Ca^{2+}]_i$ and pH_i in mediating the inotropic effect of Ang II has been proposed.[39]

A novel acid-loading mechanism has been recently reported.[12] This mechanism was proposed to exchange extracellular Cl^- for intracellular OH^- or, alternatively, to be an H^+-Cl^- symport. Whether Ang II can activate this novel mechanism through a PKC-dependent pathway was not analyzed by us. However, this possibility seems unlikely if we consider that this novel mechanism is bicarbonate independent.

The Ang II–induced increase in pH_i is not observed in the presence of HCO_3^- despite suggestions that the rise in pH_i may be a growth signal.[59,60] It would be advantageous, however, for the activated myocyte to maintain a steady-state pH_i within the normal limits but to be able to recover more rapidly from acid and alkali loads. This will be made possible through the simultaneous activation of one alkaline and one acid loader mechanism. The hypothesis that an important physiological effect of the growth factors may be to maintain a near normal pH_i while stimulating several acid-base transporters was proposed in 1989 by Boron et al.[28] This view was based on experiments studying the effects of arginine vasopressin on renal mesangial cells, and after our findings, it can be extended to the effect of Ang II on myocardium. These findings also emphasize the fact that although HEPES buffer might be a useful experimental tool, conclusions concerning the physiological processes must be drawn from experiments using a CO_2-HCO_3^- buffer–containing saline solution.[9]

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