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Signaling Pathways in the Biphasic Effect of ANG II on Na^+/H^+ Exchanger in T84 Cells

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Abstract. The effect of ANG II on pH_i, $[Ca^{2+}]_i$ and cell volume was investigated in T84 cells, a cell line originated from colon epithelium, using the probes BCECF-AM, Fluo 4-AM and acridine orange, respectively. The recovery rate of pH_i via the Na^+/H^+ exchanger was examined in the first 2 min following the acidification of pH_i with a NH_4Cl pulse. In the control situation, the pH_i recovery rate was 0.118 ± 0.001 (n = 52) pH units/min and ANG II (10^{-12} M or 10^{-9} M) increased this value (by 106% or 32%, respectively) but ANG II (10^{-7} M) decreased it to 47%. The control $[Ca^{2+}]_i$ was 99 ± 4 (n = 45) nM and ANG II increased this value in a dose-dependent manner. The ANG II effects on cell volume were minor and late and should not interfere in the measurements of pH_i recovery and $[Ca^{2+}]_i$. To document the signaling pathways in the hormonal effects we used: Staurosporine (a PKC inhibitor), W13 (a calcium-dependent calmodulin antagonist), H89 (a PKA inhibitor) or Econazole (an inhibitor of cytochrome P450 epoxygenase). Our results indicate that the biphasic effect of ANG II on Na^+/H^+ exchanger is a cAMP-independent mechanism and is the result of: 1) stimulation of the exchanger by PKC signaling pathway activation (at 10^{-12} – 10^{-7} M ANG II) and by increases of $[Ca^{2+}]_i$ in the lower range (at 10^{-12} M ANG II) and 2) inhibition of the exchanger at high $[Ca^{2+}]_i$ levels (at 10^{-9} – 10^{-7} M ANG II) through cytochrome P450 epoxygenase-dependent metabolites of the arachidonic acid signaling pathway.

Key words: Angiotensin II — Colon cells — T84 cells — Na^+/H^+ exchanger — Intracellular pH

Introduction

A dose-dependent biphasic effect of Angiotensin II (ANG II) on the Na^+/H^+ exchanger in the kidney has been reported: low concentrations of ANG II stimulate, whereas high concentrations inhibit the Na^+/H^+ exchanger in the proximal tubule (Harris & Young, 1977; Reilly, Harris & Williams, 1995; Houillier et al., 1996) and MDCK cells (a cell line with many morphological and physiological similarities with the mammalian distal nephron) (Oliveira-Souza & Mello-Aires, 2000). The present study was designed to determine whether this dual ANG II regulation of the Na^+/H^+ exchanger is also present in epithelial colon cells and to add information about the relevant signaling pathways involved in this hormonal effect. For this purpose, we evaluated the effects of both low and high doses of ANG II on the regulation of intracellular pH (pH_i) in T84 cells (a permanent cell line originated from human colon epithelium). The experiments were done in nominally HCO_3^-/CO_2–free medium, after an intracellular acid load induced by NH_4Cl, an experimental condition in which the Na^+/H^+ exchanger is the only mechanism for the pH_i regulation in T84 cells (Ramirez et al., 2000).

We studied the effect of ANG II (10^{-12}, 10^{-9} and 10^{-7} M) and/or Losartan [(10^{-6} M), an AT_1-receptor antagonist], hexa-methylene amiloride (HMA) [(0.1 mM), a specific inhibitor for the Na^+/H^+ exchanger], staurosporine [(10^{-5} M), a PKC inhibitor], W13 [(10^{-5} M), a calcium-dependent calmodulin antagonist], H89 [(10^{-4} M), a PKA inhibitor] or Econazole [(10^{-5} M), an inhibitor of cytochrome P450 epoxygenase] on pH_i recovery and on cytosolic calcium ($[Ca^{2+}]_i$). Changes in pH_i, $[Ca^{2+}]_i$ and cell volume were monitored fluorometrically by using the fluorescent probes BCECF-AM, Fluo 4-AM and acridine orange, respectively.

Our present studies indicate that in colon epithelial cells, as reported in kidney epithelial cells, ANG II has a dose-dependent biphasic effect on the Na^+/H^+ exchanger. The hormonal actions on cell volume were minor and late and should not interfere in the measurements of pH_i recovery and $[Ca^{2+}]_i$.

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The activation of the exchanger occurs through the PKC signaling pathway while its inhibition occurs via high $[Ca^{2+}]_i$ increase through cytochrome P450 epoxygenase-dependent metabolites of the arachidonic acid signaling pathway. With the consideration that ANG II exists as the tissue renin-angiotensin system in the gastrointestinal tract (Phillips, Speakman & Kimura, 1993; Sechi et al., 1993) and that the colon probably plays an important role in maintaining fluid and electrolyte homeostasis (Binder & Sandle, 1994), this dual hormonal effect we observed in T84 cells may represent a relevant mechanism of Na^+, H^+ and water transport regulation in the colon.

Materials and Methods

Cell Culture

Serial cultures of T84 cells, derived from human colon carcinoma (American Type Culture Collection, Rockville, MD; passages 46–51) were maintained in Dulbecco's modified Eagle's medium DMEM/F12 (Gibco, Grand Island, NY) supplemented with 14.5 mM $NaHCO_3$, 15 mM HEPES, 5% fetal bovine serum (Cultilab, Campinas, SP), 50.000 IU/l penicillin G and 100 mg/l streptomycin, adjusted to pH 7.4 (Dharmsathaphorn et al., 1984). The cultivated cells were kept in a CO_2 incubator (Lab-Line Instruments, Melrose Park, IL) at 37°C and 5% CO_2. After confluence was reached, the monolayer was treated with 0.05% trypsin, 0.53 mM EDTA and phosphate buffer (PBS) to resuspend the cells and to transfer them to new flasks for further multiplication or to cultivate them on glass cover slides (1.3 × 2.4 cm) for utilization in the experiments.

Measurement of pH_i by Fluorescence Microscopy

The pH_i was monitored when the cells were largely confluent, with only small discontinuities, using the fluorescent probe 2′, 7′-bi-carboxyethyl-5, 6-carboxyfluorescein acetoxy-methyl ester (BCECF-AM) (Ramirez et al., 2000). Briefly, the cells were loaded by exposure for 30 min to 12 μM BCECF-AM in solution 1 (Table 1). After the loading period, the glass coverslips were placed into a thermo-regulated chamber mounted on an inverted epifluorescence microscope (Nikon, TMD). The measured area under the microscope had a diameter of 260 μm and contained on the order of 250 cells. The coverslips remained in a fixed position, so that the same cells were studied throughout the experiment. All experiments were performed at 37°C. The cells were alternately excited at 495 and 440 nm, and the fluorescence emission was monitored at 530 nm, using a photomultiplier-based fluorescence system (Georgia Instruments, PMT-400) at time intervals of 2.5 s, for a period of 300 ms per measurement. The 495/440 excitation ratio corresponds to a specific pH_i. At the end of each experiment, calibration of the BCECF signal was achieved by exposing the cells for 15 min to a K^+-HEPES buffer solution containing 10 μM nigericin (solution 3, Table 1), at pH adjusted in a range between 6 and 7.5.

Cell pH Recovery

The cells were first bathed with 145 mM Na^+ control solution (Solution 2, Table 1), exhibiting the basal pH_i. After the acidification of pH_i by 2 min exposure to 20 mM NH_4Cl (Solution 4, Table 1) (Boron & De Weer, 1976), cell pH recovery was examined in the control situation or in presence of ANG II (10^{-12}, 10^{-9} and 10^{-7} M) alone or plus selective signaling pathway inhibitors. Since the rate of pH recovery depends on the value of cell pH achieved by the acid load (Weintraub & Machen, 1989), we used experiments in which these values were not significantly different between the studied groups (*see* Table 2). In all the experiments, we calculated the initial rate of pH_i recovery (dpH_i/dt, pH units per min) from the first 2 min of the recovery curve by linear regression analysis (*see* Figure 1). Ideally, the rate of pH_i recovery must be multiplied by the buffering capacity to give the proton flux, which is produced by the Na^+/H^+ exchanger. However, since the values of cell pH achieved by the acid load caused by ammonium (pH_i acid load, *see* Table 2) were not significantly different between the studied groups, we believe that the buffering power of the cells is the same in all our experimental groups and that pH_i variations are acceptable as estimations of the activity of the exchanger.

Measurement of $[Ca^{2+}]_i$ by Fluorescence Microscopy

In all experimental groups, changes in $[Ca^{2+}]_i$ were monitored fluorometrically by using the calcium-sensitive probe Fluo 4-AM, since the basal and non-basal levels of $[Ca^{2+}]_i$ measured from single-wavelength Fluo-4 were similar to those measured from dual-wavelength Fura-2 (Oliveira-Souza et al., 2004). Briefly, confluent cultures were loaded with 10 μM Fluo 4-AM at 37°C for 15 min and rinsed in Tyrode solution (Solution 5, Table 1) containing 0.2% bovine serum albumin (pH 7.4). Fluo-4 fluorescence intensity emitted above 505 nm was imaged by using laser excitation at 488 nm on a Zeiss LSM 510 confocal microscope. The images were recorded at time intervals of 1 s, before and after substitution of experimental solutions, for a total of 200 s. For each experiment the maximum fluorescent signal for 5 cells was averaged and then used for $[Ca^{2+}]_i$ calculation. Transformation of the fluorescent signal to $[Ca^{2+}]_i$ was performed by calibration with ionomycin (30 μM) (maximum Ca^{2+} concentration) followed by EGTA (2.5 mM) (minimum Ca^{2+} concentration) according to the Grynkiewicz equation (Grynkiewicz, Poenie & Tsien, 1985).

Measurements of Cell Volume

Changes in T84 cell volume were determined by using the fluorescent probe 10 μM acridine orange (Tararthuch et al., 2002). The area of the cells was measured at 1 min intervals for a total period of 16 min, in control conditions or during superfusion of (10^{-12} or 10^{-7} M) ANG II.

Solutions and Reagents

The composition of the solutions utilized is described in Table 1. These solutions had an osmolality of about 290 mOsmol/kg H_2O, which is the value found in the culture medium used for these cells. This osmolality was used to avoid changes of volume when the cells were transferred from the culture medium to the experimental solutions. Fluo-4/AM and BCECF-AM were purchased from Molecular Probes (Eugene, OR). ANG II (molecular weight 1.046), as well as all other applied chemicals, were obtained from Sigma Chemical Company (St. Louis, MO).

Statistics

The results are presented as means ± SEM; n is the number of experiments. Data were analyzed statistically by analysis of variance followed by the Bonferroni contrast test. Differences were considered significant if $P < 0.05$.

Table 1. Composition of solutions

	Solution $n°$ 1 (BCECF)	Solution $n°$ 2 (Control)	Solution $n°$ 3 (Nigericin)	Solution $n°$ 4 (NH_4Cl)	Solution $n°$ 5 (Tyrode)
NaCl	100	145	20.0	121.0	137
KCl	5.0	5.4	130	5.4	2.68
$CaCl_2$	1.0	1.0	1.0	1.0	1.36
KH_2PO_4	—	0.4	—	0.4	—
$MgCl_2$	1.0	0.5	1.0	0.5	0.49
$MgSO_4$	—	0.4	—	0.4	—
Na_2HPO_4	—	0.3	—	0.3	0.36
HEPES	5.0	10.0	5.0	10.0	—
Glucose	—	0.6	—	0.6	5.6
NH_4Cl	—	—	—	20.0	—
Nigericin	—	—	0.01	—	—
$NaHCO_3$	—	—	—	—	12
BCECF	0.01	—	—	—	—
PH	7.2	7.4	7.5;7.0;6.5;6.0	7.4	7.4

Values are expressed in mM. HCl or NaOH was used to titrate to the appropriate pH.

Table 2. Summary of pH_i responses in T84 cells to addition of different agents after acute acid load

	Basal pH_i	pH_i with NH_4Cl	pH_i after acid load	pH_i Recovery	dpH_i/min	n
Control	7.19 ± 0.02	7.62 ± 0.03	6.64 ± 0.04	7.06 ± 0.04	0.118 ± 0.001	52
ANG II						
(10^{-12} M)	7.23 ± 0.03	7.63 ± 0.05	6.62 ± 0.06	7.00 ± 0.03	0.243 ± 0.010*	41
(10^{-9} M)	7.18 ± 0.02	7.58 ± 0.05	6.51 ± 0.06	6.98 ± 0.06	0.156 ± 0.012*	42
(10^{-7} M)	7.17 ± 0.04	7.59 ± 0.06	6.57 ± 0.05	6.82 ± 0.08 $^{-}$	0.055 ± 0.009*	40
HMA (10^{-4} M)	7.18 ± 0.01	7.62 ± 0.02	6.61 ± 0.02	6.65 ± 0.02 $^{-}$	0.004 ± 0.003*	5
+ ANG II (10^{-12} M)	7.17 ± 0.02	7.58 ± 0.01	6.59 ± 0.01	6.62 ± 0.01 $^{-}$	0.005 ± 0.004$^{\&}$	5
+ ANG II (10^{-9} M)	7.18 ± 0.01	7.68 ± 0.01	6.63 ± 0.01	6.64 ± 0.01 $^{-}$	0.005 ± 0.004$^{\#}$	5
+ ANG II (10^{-7} M)	7.20 ± 0.09	7.60 ± 0.03	6.66 ± 0.03	6.67 ± 0.01 $^{-}$	0.004 ± 0.003$^{@}$	5
Losartan (10^{-6} M)	7.17 ± 0.05	7.68 ± 0.04	6.59 ± 0.05	7.03 ± 0.08	0.107 ± 0.006	6
+ ANG II (10^{-12} M)	7.21 ± 0.03	7.60 ± 0.03	6.62 ± 0.06	7.05 ± 0.03	0.121 ± 0.009$^{\&}$	6
+ ANG II (10^{-9} M)	7.16 ± 0.08	7.65 ± 0.05	6.53 ± 0.08	7.02 ± 0.07	0.115 ± 0.005$^{\#}$	5
+ ANG II (10^{-7} M)	7.20 ± 0.06	7.59 ± 0.07	6.57 ± 0.06	7.00 ± 0.05	0.106 ± 0.008$^{@}$	6
Staurosporine (10^{-5} M)	7.18 ± 0.02	7.62 ± 0.08	6.55 ± 0.03	7.03 ± 0.03	0.116 ± 0.010	6
+ ANG II (10^{-12} M)	7.19 ± 0.02	7.60 ± 0.13	6.60 ± 0.02	7.00 ± 0.05	0.168 ± 0.010$^{\&}$	7
+ ANG II (10^{-9} M)	7.15 ± 0.04	7.63 ± 0.07	6.52 ± 0.04	7.04 ± 0.03	0.125 ± 0.006$^{\#}$	8
+ ANG II (10^{-7} M)	7.19 ± 0.03	7.65 ± 0.09	6.61 ± 0.03	6.86 ± 0.04	0.060 ± 0.009	7
W13 (10^{-5} M)	7.15 ± 0.04	7.59 ± 0.12	6.66 ± 0.03	7.00 ± 0.03	0.108 ± 0.008	6
+ ANG II (10^{-12} M)	7.20 ± 0.05	7.62 ± 0.05	6.70 ± 0.02	6.96 ± 0.05	0.174 ± 0.015$^{\&}$	8
+ ANG II (10^{-9} M)	7.19 ± 0.02	7.65 ± 0.04	6.65 ± 0.04	6.98 ± 0.03	0.185 ± 0.007$^{\#}$	6
+ ANG II (10^{-7} M)	7.17 ± 0.03	7.60 ± 0.05	6.68 ± 0.03	7.04 ± 0.04	0.168 ± 0.007$^{@}$	7
Staurosporine + W13	7.16 ± 0.06	7.63 ± 0.09	6.58 ± 0.08	7.04 ± 0.07	0.100 ± 0.008	6
+ ANG II (10^{-12} M)	7.19 ± 0.08	7.67 ± 0.11	6.69 ± 0.10	7.10 ± 0.05	0.112 ± 0.007$^{\&}$	5
+ ANG II (10^{-9} M)	7.21 ± 0.04	7.68 ± 0.09	6.70 ± 0.09	7.05 ± 0.05	0.106 ± 0.009$^{\#}$	5
+ ANG II (10^{-7} M)	7.20 ± 0.07	7.70 ± 0.13	6.63 ± 0.07	7.00 ± 0.04	0.120 ± 0.006$^{@}$	6
H89 (10^{-4} M)	7.17 ± 0.04	7.61 ± 0.08	6.55 ± 0.07	7.03 ± 0.07	0.115 ± 0.008	8
+ ANG II (10^{-12} M)	7.18 ± 0.02	7.58 ± 0.12	6.50 ± 0.05	7.15 ± 0.03	0.238 ± 0.015	8
+ ANG II (10^{-9} M)	7.20 ± 0.03	7.65 ± 0.05	6.61 ± 0.04	7.13 ± 0.05	0.155 ± 0.008	7
+ ANG II (10^{-7} M)	7.16 ± 0.05	7.56 ± 0.09	6.53 ± 0.05	6.88 ± 0.05	0.058 ± 0.006	10
Econazole (10^{-5} M)	7.17 ± 0.06	7.62 ± 0.10	6.56 ± 0.05	6.96 ± 0.05	0.105 ± 0.015	6
+ ANG II (10^{-12} M)	7.20 ± 0.03	7.70 ± 0.08	6.61 ± 0.02	7.05 ± 0.02	0.223 ± 0.016	6
+ ANG II (10^{-9} M)	7.18 ± 0.05	7.68 ± 0.04	6.63 ± 0.06	7.01 ± 0.04	0.170 ± 0.017	5
+ ANG II (10^{-7} M)	7.15 ± 0.05	7.66 ± 0.04	6.65 ± 0.06	7.02 ± 0.04	0.175 ± 0.020$^{@}$	7

Values are means ± SE; n, $n°$ observations. dpH_i/min, pH_i recovery rate in the first 2 min.
$^{+}P < 0.05$ vs. respective basal pH_i; $^{*}P < 0.05$ vs. Control dpH_i/min: $^{\&}P < 0.05$ vs. ANG II 10^{-12} M dpH_i/min; $^{\#}P < 0.05$ vs. ANG II 10^{-9} M dpH_i/min; $^{@}P < 0.05$ vs. ANG II 10^{-7} M dpH_i/min.

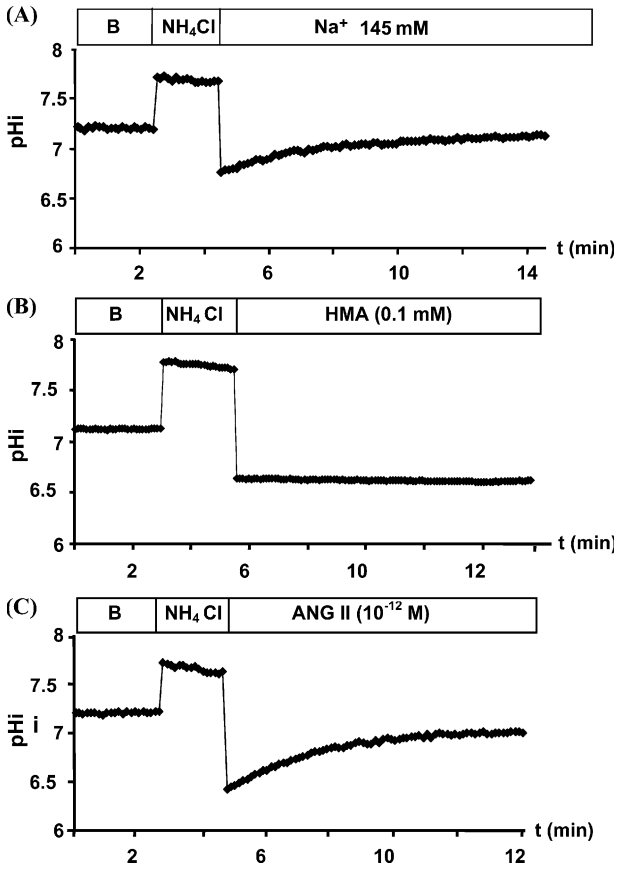

Fig. 1. Representative pH_i measurements of three different experimental groups after cellular acidification with the NH_4Cl pulse technique in T84 cells. (*A*) In the presence of 145 mM extracellular Na^+, the initial fall in pH_i is followed by a recovery of pH_i towards the basal value *B*. (*B*) In presence of external hexamethylene amiloride (HMA, 0.1 mM, a specific inhibitor of the Na^+/H^+ exchanger), the pH_i recovery rate was inhibited and the final pH_i was significantly different from the basal value, indicating that the Na^+/H^+ exchanger is the only mechanism of pH recovery in the absence of CO_2/HCO_3^-. (*C*) The addition of ANG II (10^{-12} M) to the bath caused a significant increase in the velocity of pH_i recovery towards the basal value (*see* text).

Results

pH_i

Figure 1 shows representative pH_i measurements from three different experimental groups. The cells were first bathed with control solution, exhibiting the basal pH_i. After a 2 min exposure to NH_4Cl, during which cell pH_i increased, NH_4Cl removal caused a rapid acidification of pH_i as a result of NH_3 efflux. Figure 1*A* indicates that, in the presence of external 145 mM Na^+ the initial fall in pH_i was followed by a recovery of pH_i towards the basal value. Figure 1*B* indicates that in presence of external HMA (0.1 mM, a specific inhibitor of the Na^+/H^+ exchanger) the pH_i recovery was entirely abolished, confirming that the Na^+/H^+ exchanger is the only mechanism of pH recovery in the absence of HCO_3^-/CO_2. Figure 1*C* indicates that the addition of ANG II (10^{-12} M) to the bath caused a significant increase in the velocity of pH_i recovery towards the basal value.

Table 2 summarizes the main values of pH_i responses found in all the studied experimental groups. Our results indicate that T84 cells in pH 7.4 HCO_3^--free solution have a mean baseline pH_i of 7.19 ± 0.01 (n = 352). In the control situation the pH_i recovery rate was 0.118 ± 0.001 pH units/min (n = 52) and the final pH_i was not significantly different from the basal value.

Figure 2 indicates that the addition of ANG II (10^{-12} or 10^{-9} M) to the bath caused a significant increase in the velocity of pH_i recovery (in these situations the initial fall in pH_i is followed by a recovery of pH_i towards the basal value, Table 2). However, the addition of ANG II (10^{-7} M) significantly decreased the velocity of pH_i recovery (and the final pH_i was significantly different from the basal value, Table 2). Figure 2 also indicates that HMA totally blocks the control or ANG II (10^{-12}, 10^{-9} or 10^{-7} M) pH_i recovery rates (in these situations the final pH_i was not significantly different from the acid load pH_i value, Table 2). These results indicate that in T84 cells the pH_i recovery is dependent on the Na^+/H^+ exchanger both in the control situation and in presence of ANG II.

Figure 3 shows that in the presence of Losartan (10^{-6} M) the pH_i recovery rate was not significantly different from the control value. These data indicate that Losartan has no intrinsic effects on pH_i responses. However, Losartan impairs the stimulatory effect of ANG II (10^{-12} or 10^{-9} M) and the inhibitory effect of ANG II (10^{-7} M). These results indicate that, in T84 cells, both stimulatory and inhibitory effects of ANG II on the net rate of pH_i recovery are via activation of the AT_1 receptor.

Figure 4 indicates that Staurosporine (10^{-5} M, a PKC inhibitor) alone had no effect on the pH_i recovery rate, but impaired the stimulatory effects of ANG II (10^{-12} and 10^{-9} M) and did not induce a significant change in the inhibitory effect of ANG II (10^{-7} M).

Figure 5 shows that W13 (10^{-5} M; a calcium-dependent calmodulin antagonist) alone had no effect on the pH_i recovery rate but impaired the stimulatory effect of ANG II (10^{-12} M), increased the stimulatory effect of ANG II (10^{-9} M) and reversed the inhibitory effect of ANG II (10^{-7} M). On the other hand, Fig. 6 indicates that Staurosporine plus W13 had no effect on the pH_i recovery rate, but prevented the stimulatory effects of ANG II (10^{-12} and 10^{-9} M) and the inhibitory effect of ANG II (10^{-7} M).

Taken together these results suggest a role of PKC and of a calcium-dependent calmodulin complex in regulating the rate of pH_i recovery in epithe-

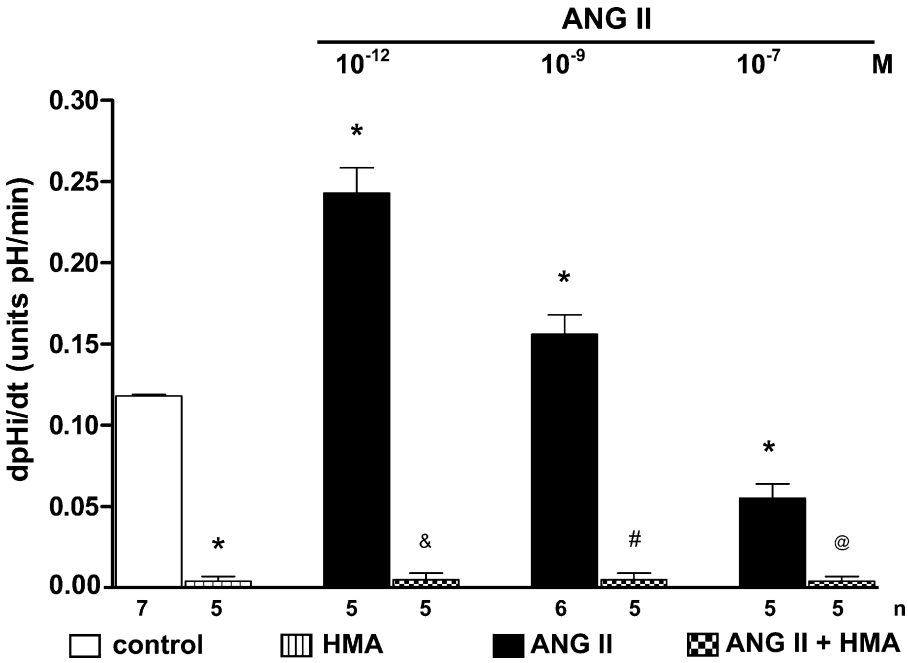

Fig. 2. Effect of ANG II (10^{-12} M, 10^{-9} M or 10^{-7} M) alone or plus hexamethylene amiloride (HMA, 0.1 mM, a specific inhibitor of the Na^+/H^+ exchanger) on the initial rate of pH_i recovery following acute intracellular acidification in T84 cells. $^*P < 0.05$ vs. control; $^{\&}P < 0.05$ vs. ANG II (10^{-12} M); $^{\#}P < 0.05$ vs. ANG II (10^{-9} M); $^{@}P < 0.05$ vs. ANG II (10^{-7} M).

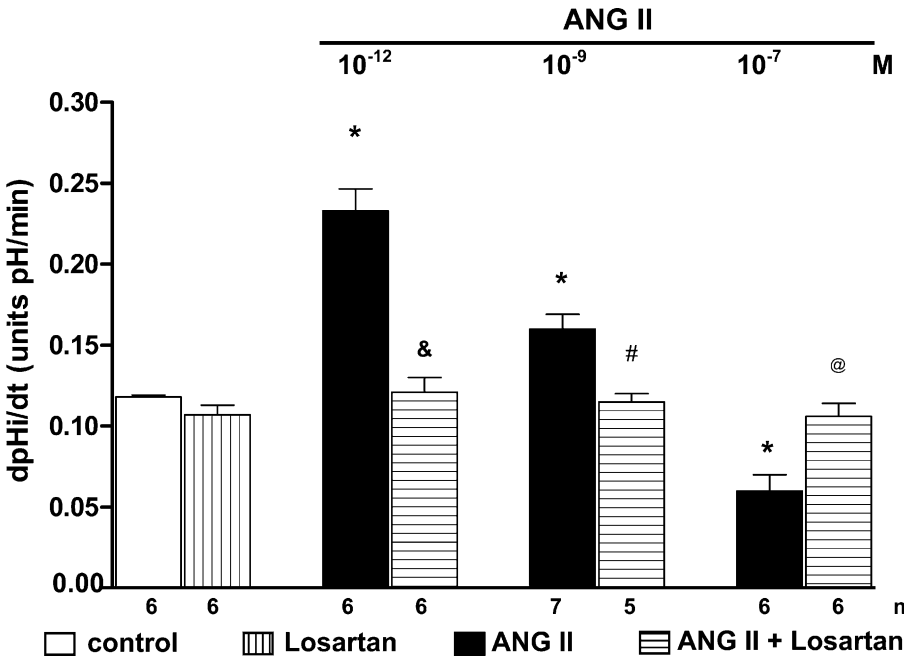

Fig. 3. Effect of Losartan (10^{-6} M, an AT_1-receptor antagonist) (alone or plus ANG II at 10^{-12} M, 10^{-9} M or 10^{-7} M) on the initial rate of pH_i recovery following acute intracellular acidification in T84 cells. $^*P < 0.05$ vs. control; $^{\&}P < 0.05$ vs. ANG II (10^{-12} M); $^{\#}P < 0.05$ vs. ANG II (10^{-9} M); $^{@}P < 0.05$ vs. ANG II (10^{-7} M).

lial colon cells, mediated by the Na^+/H^+ exchanger and stimulated/impaired by ANG II. Figure 7 shows that H89 alone (10^{-4} M, a PKA inhibitor) had no effect on the pH_i recovery rate. H89 also did not affect the stimulatory effects of ANG II (10^{-12} or 10^{-9} M) or the inhibitory effect of ANG II (10^{-7} M). These data rule out a significant role of PKA in the observed biphasic effect of ANG II on the Na^+/H^+ exchanger in colon cells.

Figure 8 indicates that Econazole (10^{-5} M, an inhibitor of cytochrome P450 epoxygenase) had no effect on the pH_i recovery rate and did not induce significant changes on the stimulatory effect of ANG II (10^{-12} or 10^{-9} M), but reversed the inhibitory effect of ANG II (10^{-7} M) to a stimulatory effect. These results suggest that a high dose of ANG II inhibits the Na^+/H^+ exchanger activity of colon cells through cytochrome P450 epoxygenase-dependent metabolites of arachidonic acid.

$[Ca^{2+}]_i$

Figure 9 shows that the addition of ANG II (10^{-12}, 10^{-9} or 10^{-7} M) to the bath of T84 cells causes a

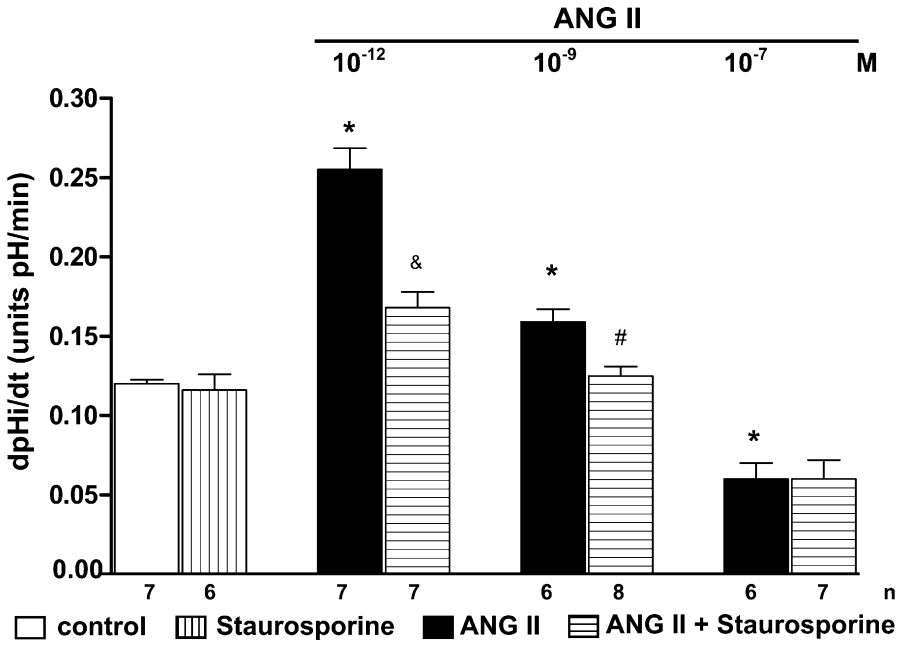

Fig. 4. Effect of Staurosporine (10^{-5} M, a PKC inhibitor) (alone or plus ANG II at 10^{-12} M, 10^{-9} M or 10^{-7} M) on the initial rate of pH_i recovery following acute intracellular acidification in T84 cells. $^{*}P < 0.05$ vs. control; $^{\&}P < 0.05$ vs. ANG II (10^{-12} M); $^{\#}P < 0.05$ vs. ANG II (10^{-9} M).

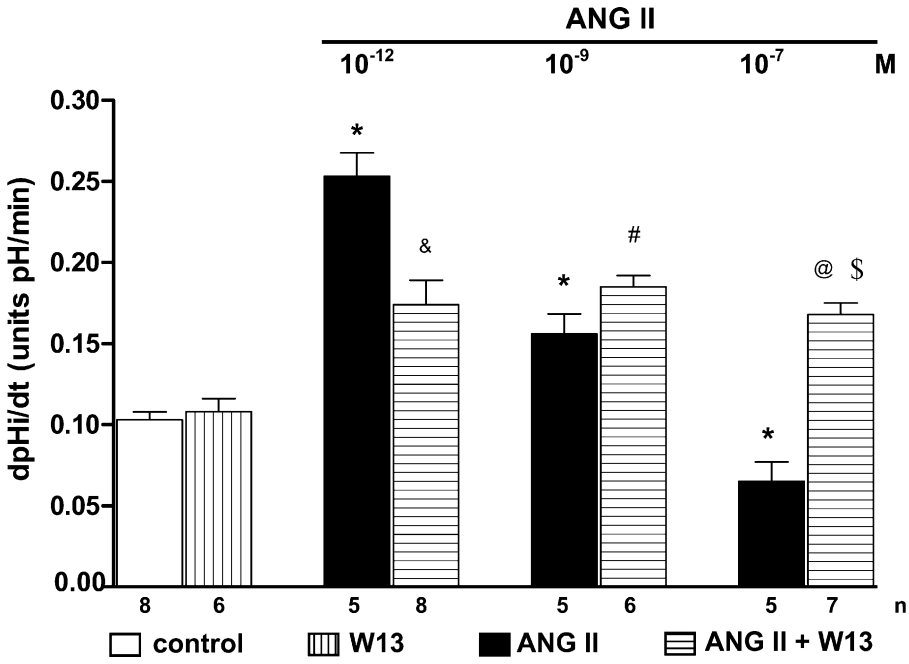

Fig. 5. Effect of W13 (10^{-5} M, a calcium-dependent calmodulin antagonist) (alone or plus ANG II at 10^{-12} M, 10^{-9} M or 10^{-7} M) on the initial rate of pH_i recovery following acute intracellular acidification in T84 cells. $^{*}P < 0.05$ vs. control; $^{\&}P < 0.05$ vs. ANG II (10^{-12} M); $^{\#}P < 0.05$ vs. ANG II (10^{-9} M); $^{@}P < 0.05$ vs. ANG II (10^{-7} M); $^{\$}P < 0.05$ vs. W13.

significant increase of the cell calcium-dependent fluorescent signal, in a dose-dependent manner. For each experiment, the maximum fluorescent signal for 5 cells was averaged and then used for calculation of $[Ca^{2+}]_i$.

Figure 10 shows that T84 cells exhibited a mean baseline $[Ca^{2+}]_i$ of 99 ± 4 (n = 45) nM. The subsequent addition of ANG II (from 10^{-12} to 10^{-7} M) increased $[Ca^{2+}]_i$ progressively from control values to 250 ± 12 (n = 36) nM. Figure 10 also shows that the addition of Staurosporine (10^{-5} M), W13 (10^{-5} M) or H89 (10^{-4} M), alone or plus ANG II, did not lead to a significant modification in $[Ca^{2+}]_i$. Econazole (10^{-5} M) alone or plus ANG II (10^{-12} M) also had no effect on $[Ca^{2+}]_i$; however, Econazole plus ANG II (10^{-9} or 10^{-7} M) caused a significant decrease in $[Ca^{2+}]_i$.

Cell Volume

Figure 11 gives the cell volume changes of three different experimental groups (with 15 cells/group). The figure shows that: in control conditions the cell volume does not change; after 3 min of superfusion (10^{-12} M) ANG II causes a small increase of cell

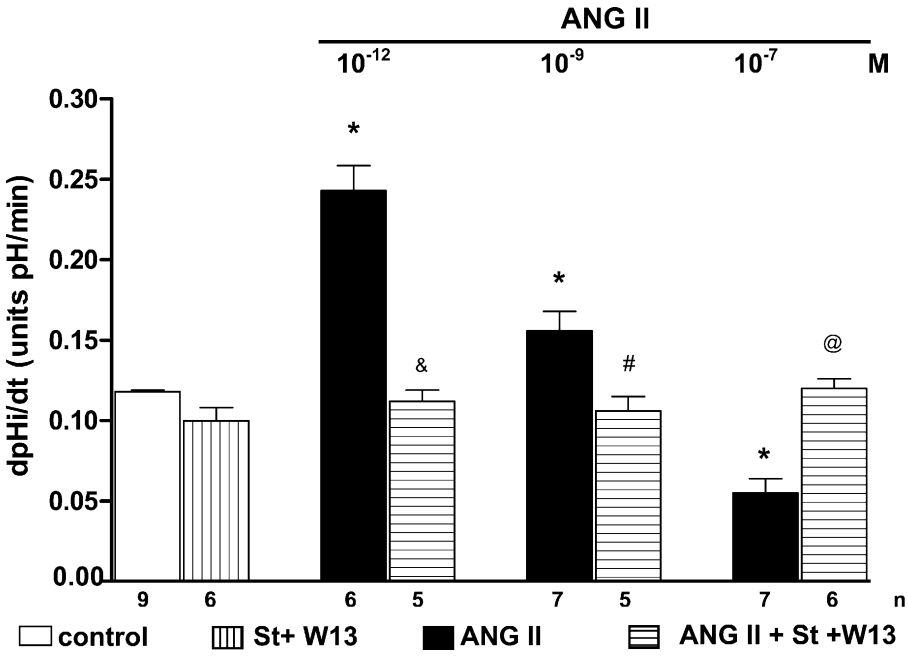

Fig. 6. Effect of Staurosporine (10^{-5} M, a PKC inhibitor) plus W13 (10^{-5} M, a calcium-dependent calmodulin antagonist) (alone or plus ANG II at 10^{-12} M, 10^{-9} M or 10^{-7} M) on the initial rate of pH_i recovery following acute intracellular acidification in T84 cells. $^{*}P < 0.05$ vs. control; $^{\&}P < 0.05$ vs. ANG II (10^{-12} M); $^{\#}P < 0.05$ vs. ANG II (10^{-9} M); $^{@}P < 0.05$ vs. ANG II (10^{-7} M).

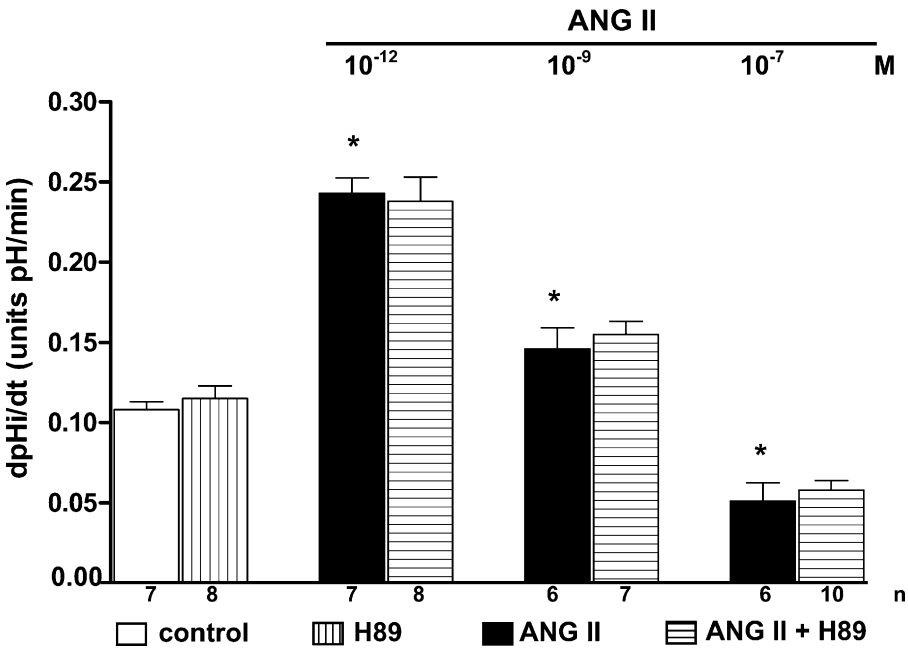

Fig. 7. Effect of H89 (10^{-4} M, a PKA inhibitor) (alone or plus ANG II at 10^{-12} M, 10^{-9} M or 10^{-7} M) on the initial rate of pH_i recovery following acute intracellular acidification in T84 cells. $^{*}P < 0.05$ vs. control.

volume; and after 6 min of superfusion (10^{-7} M) ANG II decreases the cell volume. These data indicate that the changes on cell volume are minor (at most 6%) and late and should not interfere in the measurements of pH_i recovery and $[Ca^{2+}]_i$.

Discussion

It has been shown that T84 cells, a permanent cell line derived from human colon epithelial cancer, present several properties of epithelial tissue such as polarity, intercellular connections, ion channels, volume regulation and action of peptide hormones or neurotransmitters (Dharmsathaphorn et al., 1984; Devor & Frizzell, 1998). These findings indicate that T84 cells may be used as a model for colon electrolyte transport and its regulation. In addition, it was shown that Na^+/H^+ and Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransport participate in pH_i regulation in T84 cells (Ramirez et al., 2000). However, nothing is known about the hormonal regulation of these ion transporters in T84 cells. The present study was designed to determine whether the dose-dependent

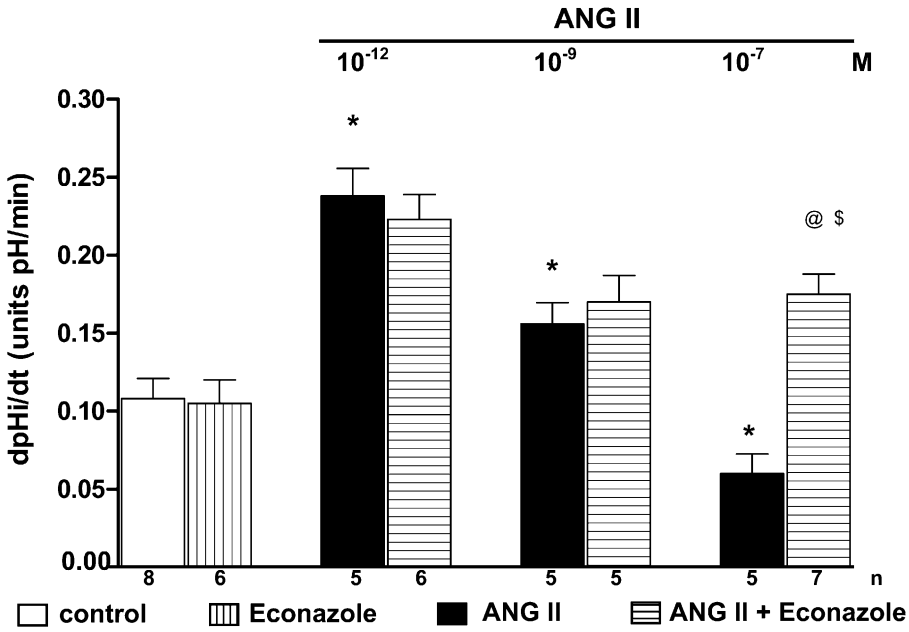

Fig. 8. Effect of Econazole (10^{-5} M, an inhibitor of cytochrome P450 epoxygenase) (alone or plus ANG II at 10^{-12} M, 10^{-9} M or 10^{-7} M) on the initial rate of pH_i recovery following acute intracellular acidification in T84 cells. *$P < 0.05$ vs. control; $^{@}P < 0.05$ vs. ANG II (10^{-7} M); $^{\$}P < 0.05$ vs. Econazole.

biphasic effect of ANG II on Na^+/H^+ exchanger, reported in kidney epithelial cells, is also present in T84 cells and to add information about the relevant signaling pathways involved in this dual hormonal effect.

Our results indicate that there are no subpopulations of cells and that all the cells respond similarly on average, since we observe that: 1) the mean baseline pH_i values of the 32 experimental groups are not significantly different (Table 2) and 2) the control pH_i recovery rates of each experimental group are not significantly different (Figs. 2 to 8). Our data also show that no pH_i recovery was observed in presence of HMA (a specific inhibitor of the Na^+/H^+ exchanger), confirming that in nominally HCO_3^-/CO_2^--free media the Na^+/H^+ exchanger is the only mechanism of pH recovery active in T84 cells (Ramirez et al., 2000).

Our data indicate that ANG II (10^{-12} or 10^{-9} M) causes a significant increase in the velocity of pH_i recovery and that ANG II (10^{-7} M) decreases it (Fig. 2). Our results also indicate that HMA totally blocks the effects of ANG II (10^{-12}, 10^{-9} or 10^{-7} M) on the pH_i recovery rate. In addition, our results indicate that both stimulatory and inhibitory effects of ANG II were prevented by simultaneous addition of Losartan, an AT_1-receptor antagonist (Fig. 3). These results are in accordance with studies indicating that in human colonic mucosa the predominant ANG II receptor subtype is AT_1 (Hirasawa et al., 2002). Our present data confirm our previous study (Musa-Aziz & Mello-Aires, 2005) about the effect of ANG II on T84 cell volume. The hormonal effects on cell volume were minor and late (after 3 min of superfusion ANG II, 10^{-12} M, causes a small increase of cell volume, and after 6 min of superfusion ANG II, 10^{-7} M, decreases the cell volume, at most 6%; Fig. 11) and should not interfere in our measurements of pH_i recovery and of $[Ca^{2+}]_i$, since we calculated these parameters, respectively, during the first 2 min or 1 to 5 seconds of superfusion with ANG II.

At the present time, we do not have information about the ANG II levels in the colon luminal compartment. Levels of ANG II in blood, on the other hand, could be similar in kidney and colon. So, it is interesting to consider: 1) similar to what has been reported in the proximal tubule, the present study indicates that in epithelial colon cells ANG II has a dose-dependent biphasic effect on Na^+/H^+ exchanger; 2) it has been documented that blocking ANG II receptors systemically decreases Na^+ and HCO_3^- absorption in proximal tubules (Liu & Cogan, 1987), which indicates that the endogenous ANG II concentrations, of picomolar range (Navar et al., 1999), upregulate Na^+ and HCO_3^- absorption in the kidney and, as our data are indicating, in T84 cells; 3) dehydration results in increased levels of ANG II, the primary effects of which are to stimulate fluid and Na^+ absorption by both the renal tubules and the intestines, thus restoring total body fluid and Na^+ content (Binder, 2003). The present experimental setup does not allow us to determine on which of the cell surfaces the observed effects of ANG II are located. However, it must be remembered that the NHE3 isoform is typically not expressed in T84 cells (Ramirez et al., 2003; Hecht et al., 2004). In addition, recently we suggested that the effects of ANG II on the regulation of pH_i in T84 cells are partly mediated by the basolateral NHE1 and the apical NHE2 isoforms (Musa-Aziz & Mello-Aires, 2005).

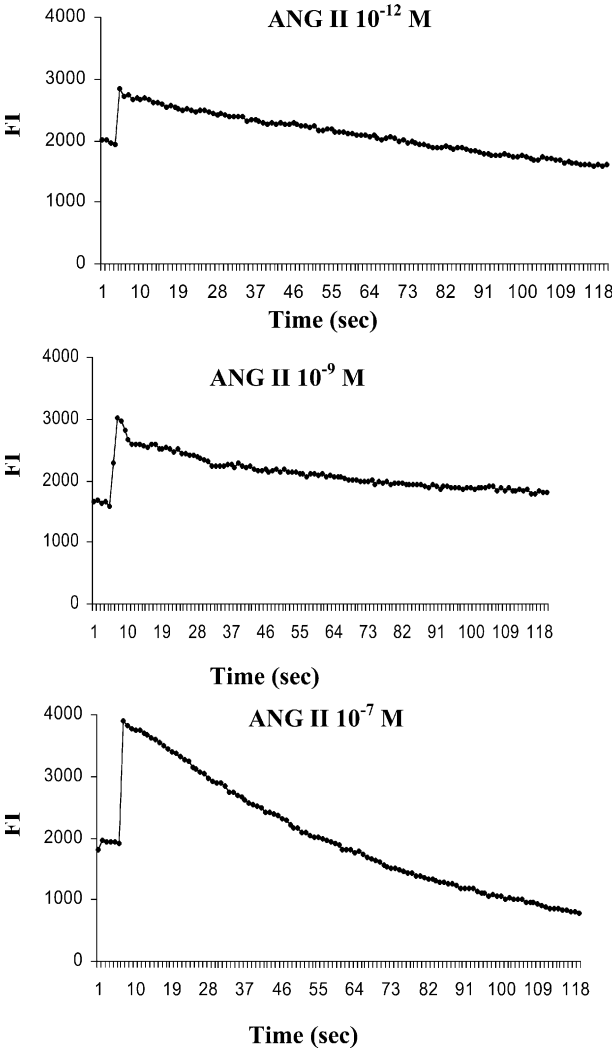

Fig. 9. Cell calcium fluorescent signal tracings during three representative experiments. The images were recorded at time intervals of 1 second, before and after addition of ANG II (10^{-12} M, 10^{-9} M or 10^{-7} M). The addition of ANG II to the bath causes a significant and dose-dependent increase of the fluorescent signal. *FI*, fluorescence intensity.

In order to determine the effects of ANG II on the V_{max} and the K_m of the mammalian Na^+/H^+ exchanger, we performed (Foster, Dudeja & Brasitus, 1986) additional experiments in MDCK cells, since it is known that this canine kidney cell line only expresses the NHE1 isoform. Our results showed that a low concentration of ANG II does not change the affinity of the exchanger for intracellular protons and therefore probably increases its expression at the plasma membrane; whereas at high concentration ANG II increases the allosteric coupling of the exchanger but decreases its expression at the plasma membrane.

To document the signaling pathways elicited by ANG II we used different inhibitors that did not show side effects on the basal pH_i, the pH_i recovery rate and the basal levels of $[Ca^{2+}]_i$ (Table 2 and Fig. 10).

Signaling Pathways Involved in the Stimulatory Effect of 10^{-12} M ANG II on the Na^+/H^+ Exchanger in T84 Cells

Two different but not exclusive signaling pathways have been proposed for the hormonal effect at this dose in renal epithelial cells: the phospholipase C (PLC)-protein kinase C (PKC) (Cano et al., 1994; Douglas & Hopper, 1994; Houillier et al., 1996) and the cAMP-protein kinase A (PKA) cascades (Romero et al., 1991; Poggioli et al., 1992; Pouysségur, 1994). Our data show that Staurosporine (a PKC inhibitor) impaired the stimulatory effect of ANG II (10^{-12} M) on the pH_i recovery rate (Fig. 4), indicating that, in T84 cells, at low dose, ANG II stimulates the Na^+/H^+ exchanger through a PKC pathway. However, in presence of Staurosporine plus ANG II (10^{-12} M) there still occured a significant increase in the velocity of pH_i recovery as compared to control (by about 42%). It has been proposed that the NHE1 exchanger has calmodulin binding sites at the cytoplasmic regulatory domain, which modulate its activity. A high-affinity site, which is tonically inhibitory of the exchanger, binds to low calcium/calmodulin suppressing the inhibition, that is, stimulating the exchanger at low Ca^{2+}/calmodulin levels; a low-affinity site, however, binds with calcium and calmodulin only at high concentrations, and under these conditions inhibits the exchanger activity (Wakabayashi et al., 1994; Wakabayashi, Shigekawa & Pouysségur, 1997). This finding is compatible with a preliminary report of the present work that has been presented in abstract form, showing that dimethyl-BAPTA-AM, impairing the path causing the increase in cell calcium, blocks both stimulatory and inhibitory effects of ANG II on Na^+/H^+ exchanger in T84 cells (Musa-Aziz & Mello-Aires, 2002). This behavior is compatible with our present data, indicating that ANG II (10^{-12} M) alone or plus Staurosporine increases $[Ca^{2+}]_i$ in the lower range (Fig. 10) and increases the velocity of pH_i recovery as compared to control. This behavior is also in accordance with our data showing that W13 (a calcium-dependent calmodulin antagonist) reduces the stimulatory effect of ANG II (10^{-12} M) on the pH_i recovery rate (Fig. 5). In addition, our experiments show that Staurosporine or W13 partly prevent the effect of ANG II (10^{-12} M) on the pH_i recovery rate, but Staurosporine plus W13 totally prevents the effect of ANG II (10^{-12} M) on this process (Fig. 6). On the other hand, Staurosporine and/or W13 do not affect $[Ca^{2+}]_i$ in the absence or presence of ANG II (10^{-12} M) (Fig. 10).

In the present study, H89 (a PKA inhibitor) plus ANG II (10^{-12} M) did not affect the pH_i recovery rate

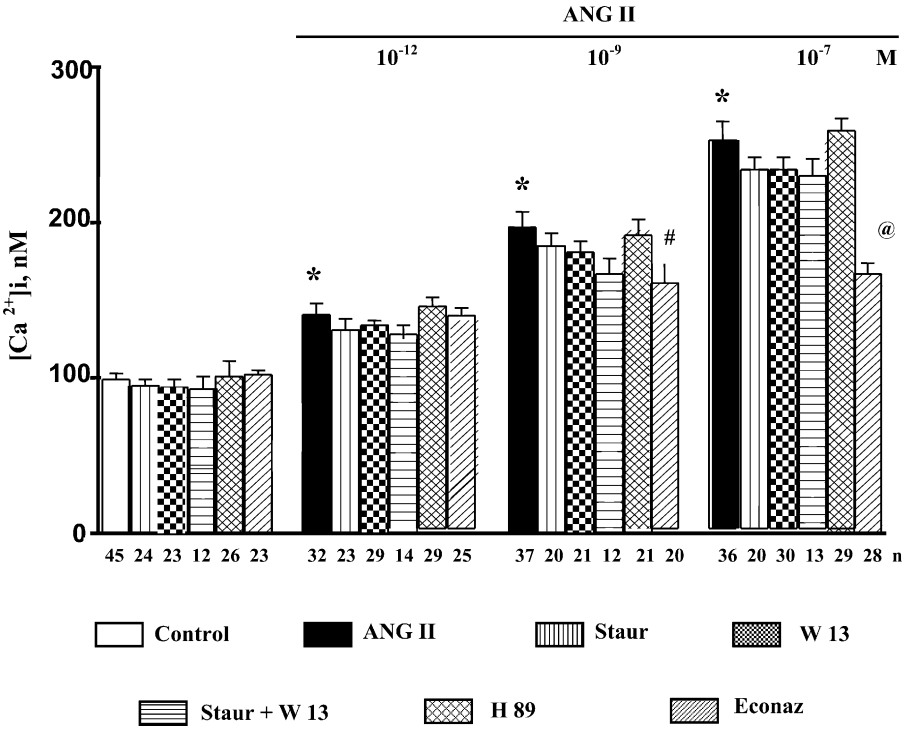

Fig. 10. Effect of ANG II (10^{-12} M, 10^{-9} M or 10^{-7} M) alone or plus Staurosporine (10^{-5} M), W13 (10^{-5} M), H89 (10^{-4} M) or Econazole (10^{-5} M) on cytosolic free calcium concentration in T84 cells. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. ANG II (10^{-9} M); $^{@}P < 0.05$ vs. ANG II (10^{-7} M).

and the $[Ca^{2+}]_i$ (Figs. 7 and 10). These data rule out a significant role of the cAMP-PKA cascade in the observed low dose ANG II stimulation of the Na^+/H^+ exchanger in T84 cells. This behavior is in accordance with data obtained in intact proximal cells (Houillier et al., 1996).

Our present results show that Econazole (an inhibitor of cytochrome P450 epoxygenase) plus ANG II (10^{-12} M) had no effect on the pH_i recovery rate and $[Ca^{2+}]_i$ (Figs. 8 and 10). These data indicate that the cytochrome P450 epoxygenase-dependent metabolites of arachidonic acid do not mediate the low-dose ANG II-induced effect.

Signaling Pathways Involved in the Stimulatory Effect of 10^{-9} M ANG II on the Na^+/H^+ Exchanger in T84 Cells

Our data show that ANG II (10^{-9} M) causes an increase in pH_i recovery rate significantly lower than the effect of ANG II (10^{-12} M) (Fig. 2). In addition, ANG II (10^{-9} M) causes an increase of $[Ca^{2+}]_i$ significantly higher than the effect of ANG II (10^{-12} M) (Fig. 10). We also found that Staurosporine alone or plus W13 impairs the stimulatory effect of ANG II (10^{-9} M) on pH_i recovery, while W13 alone increases it. Taken together, these data suggest that in T84 cells ANG II (10^{-9} M) stimulates the Na^+/H^+ exchanger through a PKC pathway and inhibits the exchanger through the binding of its low-affinity site with high calcium/calmodulin. In addition, our data indicated that ANG II (10^{-9} M) increases $[Ca^{2+}]_i$ also through cytochrome P450 epoxygenase-dependent metabolites of the arachidonic acid signaling pathway once the hormonal effect is partly inhibited by Econazole (Fig. 10). Our results also show that H89 does not induce significant changes in the effects of ANG II (10^{-9} M) on pH_i recovery and on $[Ca^{2+}]_i$ (Figs. 7 and 10). These data confirm that the cAMP-PKA cascade does not have a significant role in the observed low stimulation of the Na^+/H^+ exchanger by ANG II at 10^{-9} M. Our data are in accordance with a study (Du, Ferguson & Wang, 2003) showing that ANG II (10^{-9} M) regulates Na^+ reabsorption in rabbit proximal

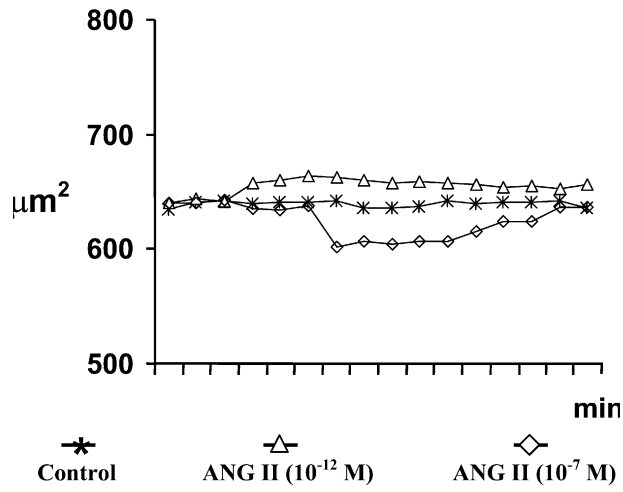

Fig. 11. Changes in T84 cell volume in control condition or during supervision ANG II (10^{-12} M or 10^{-7} M) starting at time zero. Ordinate, cell area measured by confocal microscopy. For further explanation, *see* text.

tubules by a cAMP-independent mechanism, whereas both PKC and cytosolic calcium play a critical role in modulating the effects of ANG II (10^{-9} M).

Signaling Pathways Involved in the Inhibitory Effect of 10^{-7} M ANG II on the Na^+/H^+ Exchanger in T84 Cells

In the present study, ANG II (10^{-7} M) causes a significant decrease of the pH_I recovery rate and increases $[Ca^{2+}]_i$ in the high range (Figs. 2 and 10). Two main signaling pathways have been proposed to mediate the high-dose ANG II inhibitory effect on the Na^+/H^+ exchanger in renal epithelial cells: the cytochrome P-450-dependent metabolites of arachidonic acid (Houillier et al., 1996) and the calcium-calmodulin-dependent kinase (Poggioli et al., 1992; Weinman et al., 1992).

Our data indicate that Econazole reverses the inhibitory effect of ANG II (10^{-7} M) on the Na^+/H^+ exchanger to a stimulatory effect (Fig. 8). These results confirm that, in T84 cells, at high dose, ANG II inhibits the Na^+/H^+ exchanger activity through cytochrome P450 epoxygenase-dependent metabolites of arachidonic acid. On the other hand, the finding that W13 also reversed the inhibitory effect of ANG II (10^{-7} M) to a stimulatory effect (Fig. 5) suggests that, at the cytoplasmatic regulatory domain of the NHE1 exchanger, the binding of the low-affinity site with calcium/calmodulin at high concentration plays a critical role in modulating the inhibitory hormonal effect. It is interesting to consider that Econazole not only suppressed the inhibitory effect of high-dose ANG II on the Na^+/H^+ exchanger activity but, decreasing $[Ca^{2+}]_i$ from 150% to 69% above the control value (Fig. 10), restored the hormonal stimulatory effect on the Na^+/H^+ exchanger (probably through the binding of its high-affinity site with low calcium/calmodulin). However, W13 also reversed the inhibitory effect of ANG II (10^{-7} M) on Na^+/H^+ exchanger activity, but had no effect on $[Ca^{2+}]_i$ (Figs. 5 and 10). Thus, in the presence of a calcium-dependent calmodulin antagonist, ANG II (10^{-7} M) stimulates the Na^+/H^+ exchanger in spite of high $[Ca^{2+}]_i$; these results indicate that a stimulatory effect of ANG II (10^{-7} M) is masked by an inhibitory effect. Our results show that Staurosporine does not have a significant role in the effects of ANG II (10^{-7} M) on pH_i recovery (Fig. 4); but during this experimental condition it is possible that the high inhibitory effect of ANG II (10^{-7} M), mediated by the high $[Ca^{2+}]_i$, does not permit detection of the inhibitory effect due to the inhibition by PKC. This finding is compatible with our data showing that, in presence of Staurosporine plus W13, ANG II (10^{-7} M) has no effect on the velocity of pH_i recovery as compared to control (Fig. 6).

In addition, our data show that H89 does not induce significant changes in the effects of ANG II (10^{-7} M) on pH_i recovery and on $[Ca^{2+}]_i$ (Figs. 7 and 10). These data rule out a significant role of the cAMP-PKA signaling pathway in the inhibitory effect of 10^{-7} M ANG II on the Na^+/H^+ exchanger in T84 cells.

In conclusion, our results indicate that ANG II has a dose-dependent biphasic effect on the Na^+/H^+ exchanger via activation of AT_1 receptors. This dual hormonal effect is a cAMP-independent mechanism and is the result of: 1) stimulation of the exchanger by PKC signaling pathway activation (at $10^{-12} - 10^{-7}$ M ANG II) and by increases of $[Ca^{2+}]_i$ in the lower range (at 10^{-12} M ANG II) and 2) inhibition of the exchanger at high $[Ca^{2+}]_i$ levels (at $10^{-9} - 10^{-7}$ M ANG II) through cytochrome P450 epoxygenase-dependent metabolites of the arachidonic acid signaling pathway. This finding is compatible with the indication of two sites on the C-terminal of the Na^+/H^+ exchanger, one stimulating Na^+/H^+ activity at low $[Ca^{2+}]_i$ levels, and the other inhibiting this activity at high $[Ca^{2+}]_i$. With the consideration that ANG II exists as the tissue renin-angiotensin system in the gastrointestinal tract (Sechi et al., 1993; Phillips, Speakman & Kimura, 1993) and that the colon probably plays an important role in maintaining fluid and electrolyte homeostasis (Binder & Sandle, 1994), the biphasic effect of ANG II on the Na^+/H^+ exchanger we observed in T84 cells may represent a relevant mechanism of Na^+, H^+ and water transport regulation in the colon.

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