Journal of Steroid Biochemistry & Molecular Biology 128 (2012) 89–97

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Action of ANP on the nongenomic dose-dependent biphasic effect of aldosterone on NHE1 in proximal S3 segment

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ARTICLE INFO

Article history:
Received 1 August 2011
Received in revised form
18 November 2011
Accepted 19 November 2011

Keywords:
ANP
Aldosterone
NHE1
Proximal tubule
pHi
$[Ca^{2+}]_i$

ABSTRACT

The rapid (2 min) nongenomic effects of aldosterone (ALDO) and/or spironolactone (MR antagonist), RU 486 (GR antagonist), atrial natriuretic peptide (ANP) and dimethyl-BAPTA (BAPTA) on the intracellular pH recovery rate (pHirr) via NHE1 (basolateral Na^+/H^+ exchanger isoform), after the acid load induced by NH_4Cl, and on the cytosolic free calcium concentration ($[Ca^{2+}]_i$) were investigated in the proximal S3 segment isolated from rats, by the probes BCECF-AM and FLUO-4-AM, respectively. The basal pHi was 7.15 ± 0.008 and the basal pHirr was 0.195 ± 0.012 pH units/min (number of tubules/number of tubular areas = 16/96). Our results confirmed the rapid biphasic effect of ALDO on NHE1: ALDO (10^{-12} M) increases the pHirr to approximately 59% of control value, and ALDO (10^{-6} M) decreases it to approximately 49%. Spironolactone did not change these effects, but RU 486 inhibited the stimulatory effect and maintained the inhibitory effect. ANP (10^{-6} M) or BAPTA (5×10^{-5} M) alone had no significant effect on NHE1 but prevented both effects of ALDO on this exchanger. The basal $[Ca^{2+}]_i$ was 104 ± 3 nM (15), and ALDO (10^{-12} or 10^{-6} M) increased the basal $[Ca^{2+}]_i$ to approximately 50% or 124%, respectively. RU 486, ANP and BAPTA decreased the $[Ca^{2+}]_i$ and inhibited the stimulatory effect of both doses of ALDO. The results suggest the involvement of GR on the nongenomic effects of ALDO and indicate a pHirr-regulating role for $[Ca^{2+}]_i$ that is mediated by NHE1, stimulated/impaired by ALDO, and affected by ANP or BAPTA with ALDO. The observed nongenomic hormonal interaction in the S3 segment may represent a rapid and physiologically relevant regulatory mechanism in the intact animal under conditions of volume alterations.

1. Introduction

Recently (over the past seven years), the genomic and nongenomic effects of ALDO on the Na^+/H^+ exchanger of the proximal tubule have been demonstrated [1–4], including a biphasic effect on this transporter in which low doses stimulate and high doses inhibit it [5]. The genomic effects (observed with chronic treatment with ALDO) were sensitive to spironolactone and, therefore, involve the binding of this hormone with its classic receptor (MR) [1,3–6]. However, the receptor and the signal transduction cascades involved in the nongenomic modulation of the Na^+/H^+ exchanger by ALDO need to be clarified. Studies in several cell types and in tubular segments indicate that ERK1/2, PKC and $[Ca^{2+}]_i$ participate in this process [5,7–10].

ANP inhibits the proximal [11–13] and distal reabsorption of fluid [14,15], with cyclic guanosine monophosphate (cGMP) as a second messenger [14]. In the rat proximal tubule, ANP inhibits the sodium [16,17] and bicarbonate [18] reabsorption stimulated by low doses of angiotensin II (ANG II). Studies in MDCK cells demonstrated that ANP abolishes the stimulatory and inhibitory effects of ANG II [19] or arginine vasopressin (AVP) [20] on the Na^+/H^+ exchanger and their stimulatory effects on $[Ca^{2+}]_i$. Taken together, these data suggest that there may be some interaction between these vasoactive peptide hormones in the regulation of extracellular volume.

Given the recently described genomic and nongenomic actions of ALDO in the mechanism of regulation of pHi and $[Ca^{2+}]_i$ in the S3 segment [5] and considering that the physiological doses of ALDO in blood are 10^{-10} to 10^{-9} M and that they can increase or decrease in conditions of extracellular volume modification, the objective of the present study was to examine the mechanism of interaction between the nongenomic effects of ALDO (10^{-12} or 10^{-6} M, 2 min preincubation) and ANP (10^{-6} M) or BAPTA (5×10^{-5} M) on the NHE1 exchanger and $[Ca^{2+}]_i$ in this portion of the proximal tubule of rat kidneys.

Abbreviations: ALDO, Aldosterone; ANP, atrial natriuretic peptide; BAPTA, dimethyl-BAPTA; pHi, intracellular pH; pHirr, intracellular pH recovery rate; NHE1, basolateral Na^+/H^+ exchanger isoform; $[Ca^{2+}]_i$, cytosolic free calcium concentration.

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doi:10.1016/j.jsbmb.2011.11.011

Table 1
Composition of solutions.

	Solution 1 (control)	Solution 2 (K^+-HEPES)	Solution 3 (NH_4Cl)	Solution 4 ($0Na^+_e$)	Solution 5 (Tyrode's)
NaCl	140	20	120	–	137
KH_2PO_4	2.5	–	2.5	2.5	–
$CaCl_2$	1	1	1	1	1.36
$MgSO_4$	1	1	1	1	–
$MgCl_2$	–	–	–	–	0.49
Glucose	5.5	–	5	5	5.6
L-Alanine	5	–	5	5	–
HEPES	10	5	10	10	5
NH_4Cl	–	–	20	–	–
NMDG	–	–	–	140	–
KCl	–	130	–	–	2.68
$NaHCO_3$	–	–	–	–	12
Na_2HPO_4	–	–	–	–	0.36
Nigericine	–	0.01	–	–	–
pH	7.4	6.18; 7.14; 8.11	7.4	7.4	7.4

Values are expressed in mM. NMDG, *N*-methyl-D-glucamine. HCl or NaOH were used in all Na^+-containing solutions to titrate to the appropriate pH, and KOH was used in Na^+-free solution.

2. Materials and methods

2.1. Preparation of isolated S3 segment of rat

Male Wistar rats (90 g) were anesthetized by tiletamine/zolazepam (30 mg/kg) and xylazine (2 mg/kg). Their kidneys were removed and slices 2 mm in thickness were prepared. Microdissection of the tubules was performed using tweezers under a stereomicroscope in ice-cold normal Ringer solution. The S3 segments were dissected from the outer stripe of the outer medulla [21,22] and were identified as the proximal straight tubule contiguous to the thin descending limb of the loop of Henle. Then, the S3 segments were transferred to glass coverslips prepared with poly-D-lysine for tubule adhesion. The coverslips were mounted on an inverted microscope (Olympus IX70) in a thermostatically regulated perfusion chamber with solutions that were changed by means of valves.

2.2. Viability of the tubules

After the experiments, the integrity of the S3 segments was confirmed by histological analysis and trypan blue exclusion. The tubules were removed to trypan blue (0.4%) prepared in a buffered isotonic salt solution (pH 7.4). This solution (0.1 ml) was added to the bath for 3 min at room temperature, and the color of the cell cytoplasm of the tubules was observed [23].

2.3. Measurement of pHi

For digital imaging of pHi, the S3 segments were incubated in a HEPES-buffered solution with 140 mM Na^+ (control solution, Table 1) containing 12 μM BCECF-AM for 20 min at 37 °C. The pHi was calculated from the fluorescence emission ratio collected every 5 s with an intensified ICCD-350F camera during excitation at 440 and 490 nm and emission at 530 nm. The fluorescence excitation ratio, I_{490}/I_{440}, was displayed in pseudo-color on the monitor, and a maximum of 6 areas per tubule were defined for measurement. The pHi was standardized by the high K^+/nigericin (solution 2, Table 1) technique [24].

2.4. Cell pH recovery rate

After superfusion of the S3 segments with control solution alone to measure the basal pHi, the segment was induced to alkalization by 2 min of exposure to 20 mM NH_4Cl solution (solution 3, Table 1) [25], followed by acidification by the return to control solution. The pHirr was measured in the presence of 140 mM external Na^+ (solution 1), in the absence of external Na^+ (solution 4) or in the presence of the following agents: 500 nM HOE 694 (a specific inhibitor of basolateral NHE1), 4.6×10^{-8} M concanamycin (a H^+-ATPase inhibitor), 10^{-12} or 10^{-6} M ALDO and/or 10 μM spironolactone (a MR inhibitor), 10^{-6} M RU 486 (a GR inhibitor), 10^{-6} M ANP or 5×10^{-5} M BAPTA (a calcium chelator). These drugs were added to the bath at the same time as the acid pulse for a total of 2 min of preincubation. In all experiments, the pHirr (dpHi/dt, pH units/min) was calculated in the first 2 min after the start of the pHi recovery curve, by linear regression analysis. Calculations and graphical representations were performed by an Excel program after importing the results from a data-acquisition program.

2.5. Measurement of $[Ca^{2+}]_i$

The S3 segments were loaded for 15 min with 10 (M of the calcium-sensitive probe FLUO-4-AM [19] at 37 °C and rinsed in Tyrode's solution (solution 5). The FLUO-4 intensity emitted above 505 nm was imaged using laser excitation at 488 nm on a Zeiss LSM 510 confocal microscope. The images were continuously acquired (at time intervals of 2 s) before and after substitution of the experimental solutions. The intracellular calibration was performed using 2.5 mM EGTA in a Ca^{2+}-free bath and then in a 1.36 mM Ca^{2+} bath containing ionomycin (5 μM) to measure the minimum (F_{min}) and the maximum (F_{max}) cell calcium fluorescent signals, respectively. The standard equation $[Ca^{2+}]_i = K_d \times (F - F_{min})/(F_{max} - F)$ was used to calculate the experimental values of $[Ca^{2+}]_i$ [26], using the dissociation constant (K_d) of 345 nM (according to the Molecular Probes catalog).

2.6. Solutions and reagents

The solutions utilized had an osmolality of about 300 mOsmol/kg H_2O and pH 7.4. BCECF-AM and FLUO-4-AM were obtained from Molecular Probes (Eugene, OR, USA). The other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.7. Statistics

The results are presented as means ± SEM. pHirr points are given as *N*/*n*, where *N* is the number of superfused tubules, and *n* is the

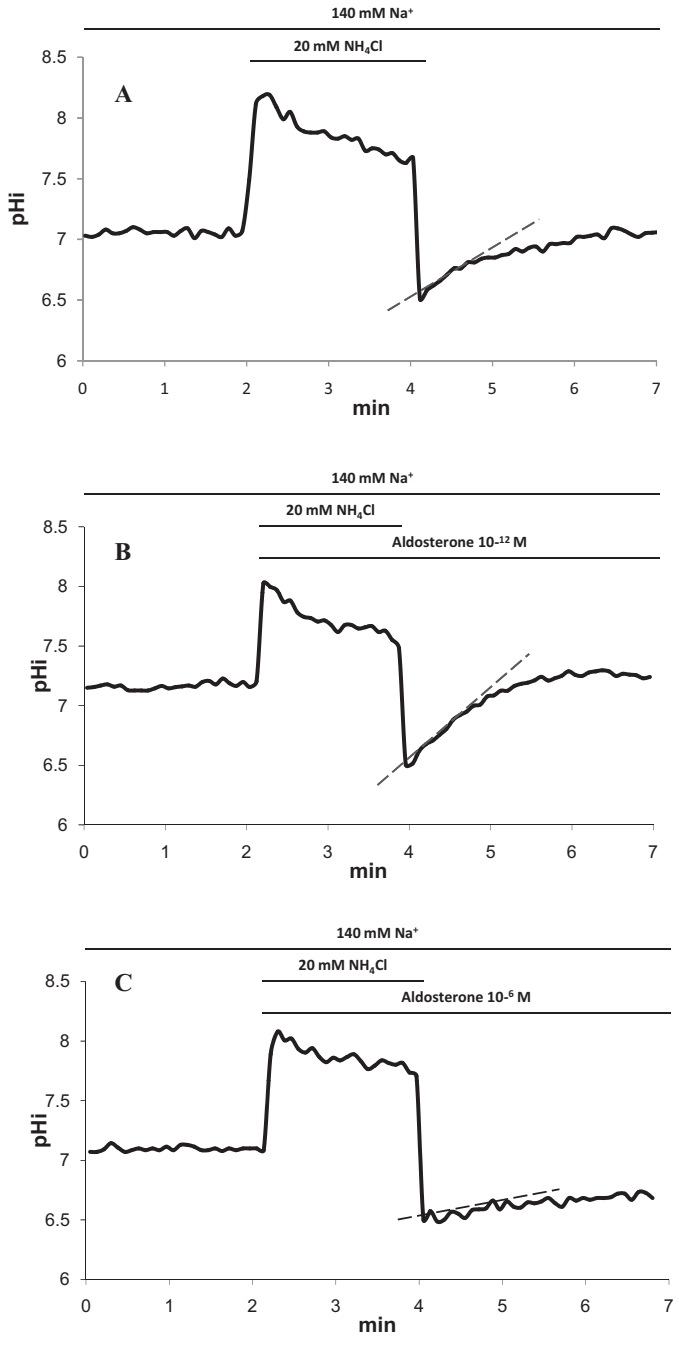

Fig. 1. Nongenomic dose-dependent biphasic effect of aldosterone (ALDO) on Na^+/H^+ exchanger activity. (A) In the presence of 140 mM Na^+ control solution, the initial decrease in pHi is followed by a recovery of pHi toward the basal value. (B) In the presence of external control solution with 10^{-12} M ALDO, the pHi recovery rate was increased. (C) In the presence of external Na^+ control solution with 10^{-6} M ALDO, the pHi recovery rate was decreased (and the final pHi was different from the basal value).

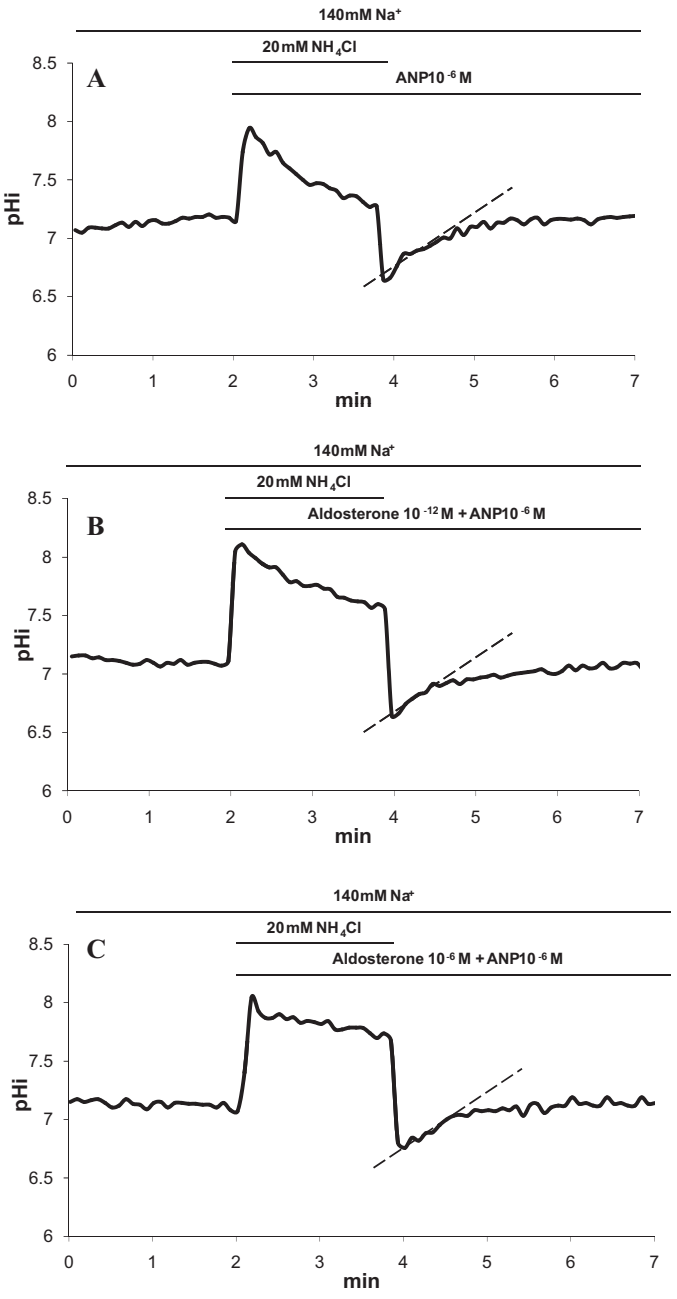

Fig. 2. ANP inhibits the nongenomic biphasic effect of aldosterone (ALDO) on Na^+/H^+ exchanger. (A) In the presence of 10^{-6} M ANP alone, the pHi recovery rate was not different from the control. (B) In the presence of ANP with 10^{-12} M ALDO, the pHi recovery was not different from the control because ANP abolished the stimulatory effect of 10^{-12} M ALDO on the velocity of pHi recovery. (C) In the presence of ANP with 10^{-6} M ALDO, the pHi recovery also was not different from the control because ANP abolished the inhibitory effect of 10^{-6} M ALDO on the velocity of pHi recovery.

number of measured areas; the means were calculated from *N*, the number of tubules. $[Ca^{2+}]_i$ points are given as *N*, where *N* is the number of tubules (each tubule is the average of 10 cell areas). Data were analyzed statistically by analysis of variance followed by the Bonferroni's contrast test. Differences were considered significant if $P < 0.05$.

This study was approved by the Biomedical Sciences Institute/USP–Ethical Committee for Animal Research (CEEA).

3. Results

3.1. Intracellular pH

The results indicate that the S3 segment in the absence of bicarbonate and presence of 140 mM Na^+ control solution has a mean basal pHi of 7.15 ± 0.008 (16/96) (Table 2).

Fig. 1A shows a representative experiment in which S3 segments were first bathed with control solution to exhibit the basal pHi. During the 2 min exposure to NH_4Cl, the pHi increased transiently, and the removal of NH_4Cl caused a rapid acidification of pHi. Then, with

Table 2
Summary of pHi measurements in presence of 140 mM external Na^+ control solution alone or with HOE 694, 0 external Na^+ solution alone or with Concanamycin, ALDO and/or spironolactone, RU 486, ANP or BAPTA.

	Basal pHi	pHi acid load	Final pHi	dpHi/dt (pH units/min)	*N*/*n*
Control	7.15 ± 0.008	6.55 ± 0.084	7.12 ± 0.045	0.195 ± 0.012	16/96
HOE 500 nM	7.03 ± 0.018	6.22 ± 0.125	6,16 ± 0.169[a]	−0.042 ± 0.018[a]	5/27
0 Na^+_e	7.12 ± 0.020	6.0 ± 0.058	6,40 ± 0.068[a]	0.068 ± 0.008[a]	8/27
0 Na^+_e + Concanamicyn 4.6×10^{-8} M	7.06 ± 0.033	6.23 ± 0.038	6,15 ± 0.050[a]	−0.020 ± 0.014[a,b]	5/25
ALDO 10^{-12} M	7.11 ± 0.011	6.36 ± 0.059	7.12 ± 0.058	0.310 ± 0.026[a]	6/24
ALDO 10^{-6} M	7.12 ± 0.019	6.58 ± 0.074	6.89 ± 0.087[a]	0.096 ± 0.009[a]	5/18
Spironolactone 10 μM	7.08 ± 0.022	6.67 ± 0.046	7.02 ± 0.036	0.192 ± 0.006	6/30
Spironolactone + ALDO 10^{-12} M	7.07 ± 0.017	6.33 ± 0.044	7.07 ± 0.014	0.296 ± 0.008[a]	5/15
Spironolactone + ALDO 10^{-6} M	7.07 ± 0.012	6.10 ± 0.045	6.41 ± 0.029[a]	0.090 ± 0.005[a]	5/24
RU 486 10^{-6} M	7.06 ± 0.010	6.11 ± 0.036	6.54 ± 0.031[a]	0.118 ± 0.002[a]	6/31
RU 486 + ALDO 10^{-12} M	7.04 ± 0.015	6.31 ± 0.019	7.03 ± 0.014[a]	0.197 ± 0.008[a,b]	6/25
RU 486 + ALDO 10^{-6} M	7.08 ± 0.008	6.19 ± 0.040	6.59 ± 0.035	0.126 ± 0.003[d]	6/24
ANP 10^{-6} M	7.14 ± 0.034	6.40 ± 0.192	7.02 ± 0.143	0.218 ± 0.012	5/23
ANP + ALDO 10^{-12} M	7.13 ± 0.010	6.80 ± 0.114	7.31 ± 0.088	0.20 ± 0.027[c]	5/15
ANP + ALDO 10^{-6} M	7.11 ± 0.012	6.61 ± 0.125	7.11 ± 0.086	0.211 ± 0.022[d]	7/25
BAPTA 5×10^{-5} M	7.13 ± 0.039	6.40 ± 0.072	7.11 ± 0.039	0.214 ± 0.015	6/16
BAPTA + ALDO 10^{-12} M	7.05 ± 0.007	6.47 ± 0.054	7.03 ± 0.008	0.216 ± 0.009[c]	7/26
BAPTA + ALDO 10^{-6} M	7.08 ± 0.019	6.36 ± 0.082	7.06 ± 0.021	0.191 ± 0.012[d]	6/16

Values are means ± SE; *N*/*n* is the number of tubules/number of areas.
[a] $P < 0.03$ vs. respective control.
[b] $P < 0.001$ vs. 0 Na^+_e.
[c] $P < 0.02$ vs. Aldo 10^{-12} M.
[d] $P < 0.008$ vs. Aldo 10^{-6} M.

the return of external control solution, this fall in pHi was immediately followed by a recovery toward the basal value. According to results previously published by our laboratory in isolated rat proximal S3 segments, this recovery of pHi occurs mainly via the Na^+/H^+ exchanger [5]. Fig. 1B indicates that the addition of ALDO at 10^{-12} M to the bath increased the pHirr. However, Fig. 1C shows that the addition of ALDO at 10^{-6} M decreased the pHirr, and pHi recovery was not complete.

Fig. 2A shows a representative experiment to indicate that with ANP (10^{-6} M) alone, the pHirr and the final pHi were not different from the control value. However, Fig. 2B and C shows that ANP impaired both the stimulatory and inhibitory effects of ALDO on

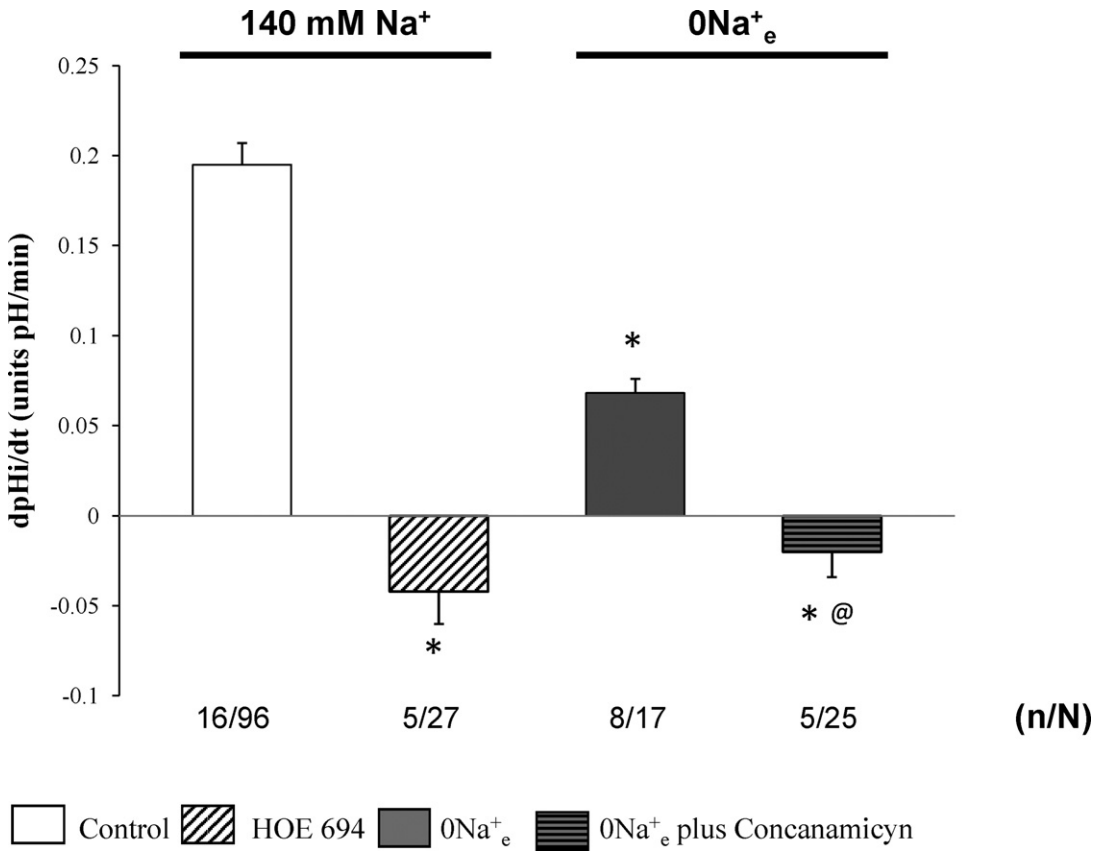

Fig. 3. Mean values of intracellular pH recovery rate after acid load. The intracellular pH recovery rate was evaluated in the presence of 140 mM external Na^+ control solution, HOE 694 or 0 external Na^+ solution alone or with concanamycin. *N*/*n* = number of tubules/number of areas. *$P < 0.001$ vs. respective control.

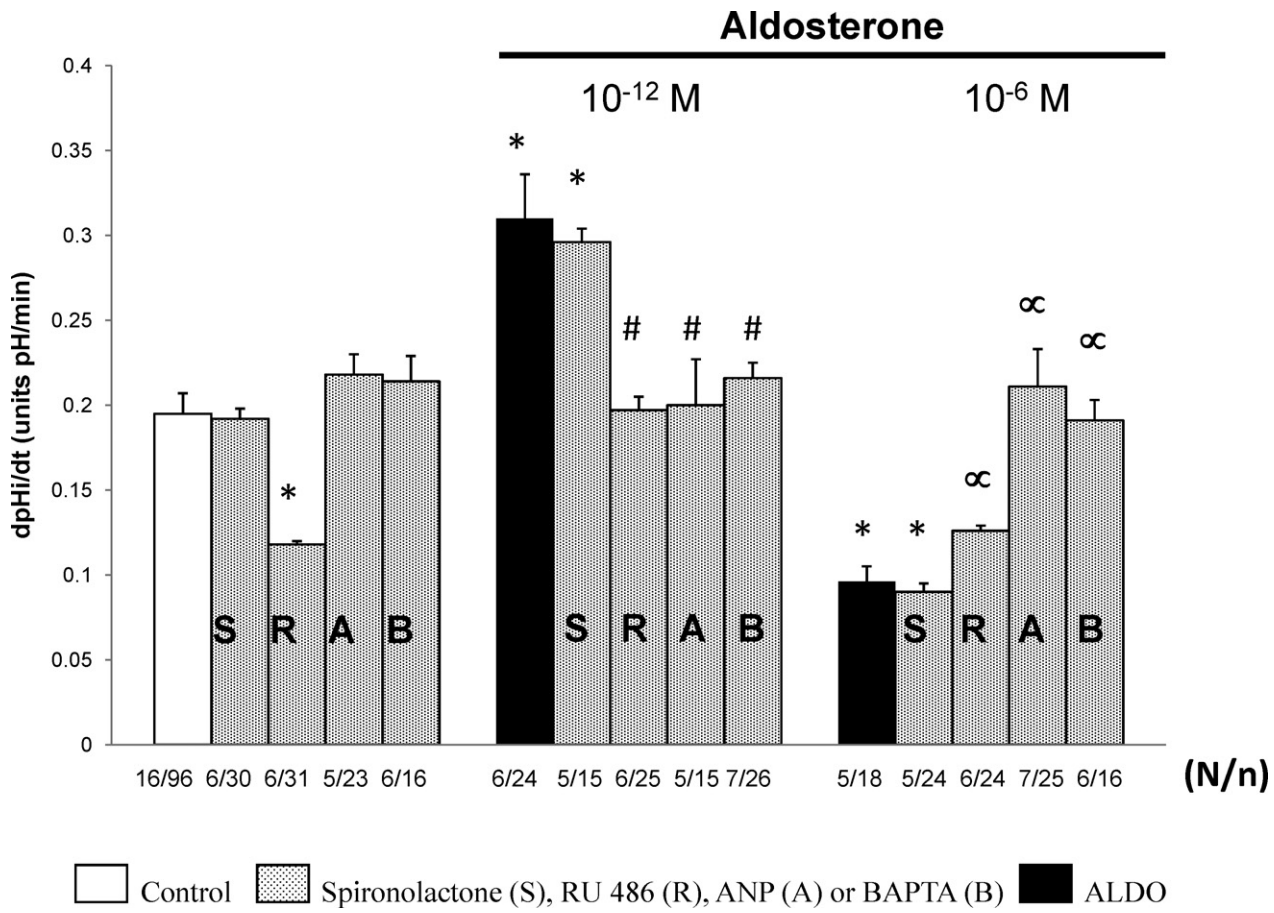

Fig. 4. Mean values of the intracellular pH recovery rate after acid load. The intracellular pH recovery rate was evaluated in the presence of ALDO (10^{-12} or 10^{-6} M) alone or with spironolactone (10 μM), RU 486 (10^{-6} M), ANP (10^{-6} M) or BAPTA (5×10^{-5} M). *N*/*n* = number of tubules/number of areas. $^{*}P < 0.005$ vs. respective control. $^{\ddagger}P < 0.02$ vs. respective control and 10^{-12} M ALDO. $^{\#}P < 0.02$ vs. 10^{-12} M ALDO. $^{\propto}P < 0.008$ vs. 10^{-6} M ALDO.

the pHirr (and during both situations, the final pHi was not different from the basal value).

Fig. 3 shows that in the control situation (140 mM Na^+_e) the mean pHirr was 0.195 ± 0.012 pH units/min (16/96), and the superfusion of tubules with HOE 694 alone inhibited the pHirr, indicating that the pHirr is mostly due to the basolateral NHE1 in S3 segments. In addition, Fig. 3 shows that in the absence of Na^+_e (a condition that inhibits the activity of Na^+/H^+ exchanger), there was a significantly lower pHirr, indicating that a Na^+-independent H^+ extrusion mechanism exists in the S3 segment of normal rats. This small pHirr was abolished by concanamycin, showing that the H^+-ATPase is the only mechanism responsible for this Na^+-independent H^+ transport. However, this mechanism of cellular extrusion of H^+ initiates about 2.5 min after cellular acidification with the NH_4Cl pulse.

Fig. 4 indicates that 10^{-12} M ALDO increased the pHirr by approximately 59% of the control value, and 10^{-6} M ALDO decreased it by approximately 49% of the control value. Spironolactone alone did not alter the pHirr and did not prevent the stimulatory and the inhibitory effects of ALDO on the Na^+/H^+ exchanger, demonstrating that this rapid biphasic effect of ALDO is independent of binding with the MR receptor. RU 486 alone decreased the pHirr (approximately 39% of control value); in addition, RU 486 abolished the stimulatory effect of ALDO but did not alter its inhibitory effect on the Na^+/H^+ exchanger. These results suggest that the GR antagonism interferes with the nongenomic stimulatory effect of ALDO on the Na^+/H^+ exchanger. Fig. 4 also shows that, compared to the control, ANP or BAPTA alone did not significantly alter the pHirr; however, ANP or BAPTA significantly abolished both stimulatory and inhibitory effects of ALDO on the pHirr.

Table 2 summarizes the mean values of pHi and pHirr responses found in all experimental groups studied.

3.2. Cytosolic calcium

Fig. 5 gives the cell calcium fluorescent signal tracing during 3 min in one representative experiment from each of 6 experimental groups. The images were continuously acquired (at time intervals of 2 s) before and after the addition of different drug solutions to the bath. The baseline value did not significantly change. However, approximately 0.4 min after the addition of ALDO (10^{-12} M or 10^{-6} M), there was a transient (approximately 1.5 min) and dose-dependent increase of the fluorescent signal, followed by a recovery toward the basal value. The addition of ANP (10^{-6} M) alone to the bath led to a rapid decrease of the fluorescent signal and prevented the dose-dependent stimulatory effect of aldosterone.

Fig. 6 and Table 3 show that the S3 segment exhibited a mean baseline $[Ca^{2+}]_i$ of 104 ± 3 nM (15). ALDO, at a concentration of 10^{-12} or 10^{-6} M, caused an increase of this parameter to approximately 50% or 124% of the control value, respectively. Spironolactone (10 μM) alone did not change the basal value of the $[Ca^{2+}]_i$ or the stimulatory effect of either dose of ALDO on the $[Ca^{2+}]_i$. RU 486 (10^{-6} M), ANP (10^{-6} M) and BAPTA (5×10^{-5} M) decreased the $[Ca^{2+}]_i$ to approximately 31%, 44% and 52% of the basal value, respectively. ANP and BAPTA also decreased the stimulatory effect of ALDO (10^{-12} or 10^{-6} M) on the $[Ca^{2+}]_i$; however, RU

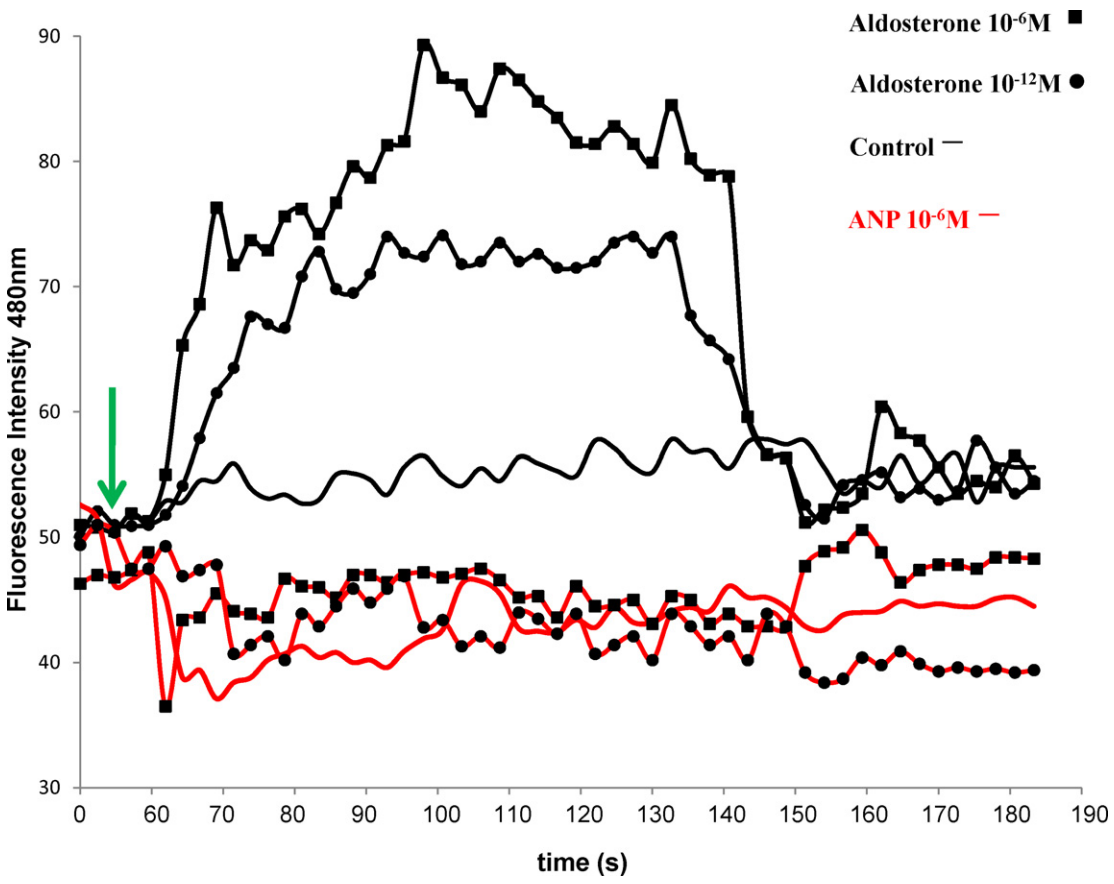

Fig. 5. Representative experiments of cell calcium fluorescent signal tracing. The images were continuously acquired in the presence of control solution or before and after (green arrow) the addition of ALDO (10^{-12} or 10^{-6} M) and/or ANP (10^{-6} M).

486 completely prevented the stimulatory effect of 10^{-12} M ALDO and reversed the stimulatory effect of 10^{-6} M ALDO to an inhibitory effect.

The histological analysis, performed after the measurement of pHirr or $[Ca^{2+}]_i$, revealed normal tubular structures with complete cubical cells and a brush-border membrane typical of proximal tubules. The tubules with up to 1 h pi at 37 °C showed no change in cytoplasmic staining, indicating the maintenance of cell membrane integrity after pHirr or $[Ca^{2+}]_i$ measurements.

Table 3
Means values of cytosolic calcium concentration in presence of control solution, ALDO and/or Spironolactone, RU 486, ANP or BAPTA.

Experimental groups	$[Ca^{2+}]_i$ (nM)	*N*
Control	104 ± 3	15
ALDO 10^{-12} M	156 ± 9[a]	5
ALDO 10^{-6} M	233 ± 9[a]	5
Spironolactone 10 μM	99 ± 5	5
Spironolactone + ALDO 10^{-12} M	166 ± 2[a]	5
Spironolactone + ALDO 10^{-6} M	235 ± 7[a]	5
RU 486 10^{-6} M	72 ± 1[a]	5
RU 486 + ALDO 10^{-12} M	100 ± 2[b]	5
RU 486 + ALDO 10^{-6} M	74 ± 1[c]	5
ANP 10^{-6} M	58 ± 2[a]	10
ANP + ALDO 10^{-12} M	80 ± 3[b]	5
ANP + ALDO 10^{-6} M	113 ± 7[d]	5
BAPTA 5×10^{-5}M	50 ± 2[a]	5
BAPTA + ALDO 10^{-12} M	78 ± 3[b]	5
BAPTA + ALDO 10^{-6} M	113 ± 1[d]	5

Values are means ± SE. *N* is the number of tubules (each tubule is the average of 10 cell areas).
[a] $P < 0.0001$ vs. respective control.
[b] $P < 0.0005$ vs. respective control and ALDO 10^{-12} M.
[c] $P < 0.0001$ vs. ALDO 10^{-6} M.
[d] $P < 0.0001$ vs. respective control and ALDO 10^{-6} M.

4. Discussion

The purpose of this study was to clarify the mechanism of interaction between the nongenomic effects (2 min preincubation) of ALDO and/or ANP on Na^+/H^+ exchanger and on $[Ca^{2+}]_i$ in isolated proximal S3 segment of rats. This is a region of the nephron where the mechanisms of tubular ion transport are less studied because it is located in the outer stripe of the outer medulla, a region difficult to access in general and impossible to access directly by *in vivo* micropuncture.

Our results indicate that in the S3 segment, the pHi recovery mostly occurs via the Na^+/H^+ exchanger, because the superfusion of the tubules with HOE 694 (a specific inhibitor of basolateral NHE1) promotes the complete inhibition of pHirr. These results are in accordance with previous data published by our laboratory [5]. Our results also indicate that during the superfusion of the S3 segment with a zero Na^+ solution (which inhibits the activity of the Na^+/H^+ exchanger), a small pHirr still was observed, which was abolished by concanamycin (a H^+-ATPase inhibitor); these data agree with recently published results [27] showing that in the S3 segment the pHi recovery also occurs via H^+-ATPase. However, in our present study and recently published work [27], the activity of this transporter begins about 2.5 min after the acid pulse and does not reach the basal pHi. Thus, this mechanism of cellular extrusion of H^+ does not interfere with the present evaluation of pHirr dependent on the

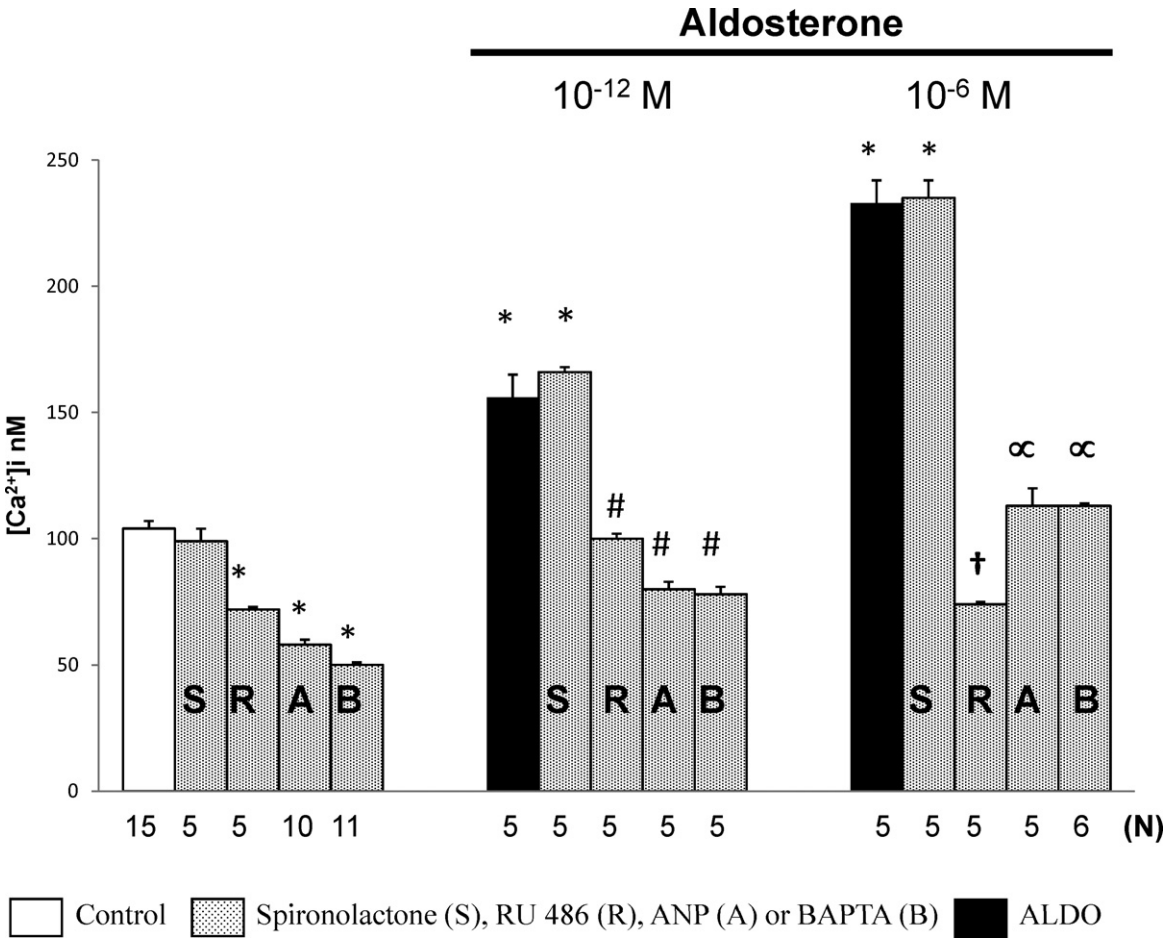

Fig. 6. Mean values of cytosolic calcium concentration. The intracellular calcium concentration was evaluated in the presence of control solution, ALDO (10^{-12} or 10^{-6} M) and/or spironolactone (10 μM), RU 486 (10^{-6} M), ANP (10^{-6} M) or BAPTA (5×10^{-5} M). *N* = number of tubules (each tubule is the average of 10 cell areas). $^{*}P < 0.0001$ vs. respective control. $^{\#}P < 0.0005$ vs. respective control and 10^{-12} M ALDO. $^{\dagger}P < 0.0001$ vs. 10^{-6} M ALDO. $^{\propto}P < 0.0001$ vs. control and 10^{-6} M ALDO.

Na^+/H^+ exchanger (because it is calculated within the first 2 min after cellular acidification).

In theory, NHE1 is expressed in the basolateral membrane of all segments of the renal tubule, where it is involved with cytoplasmic pH and volume regulation, and is considered an important target of ALDO nongenomic actions [28,29]. In our present studies, we observed a rapid effect of ALDO on NHE1. Similar results were reported by other authors [7,8,30–32], who propose that such effects occur through a nongenomic pathway. Our previous experiments [5], also in the S3 segment of rats, showed that the effects of ALDO (with 2 or 15 min of preincubation) on the NHE1 exchanger isoform occur through a nongenomic pathway because they were insensitive to actinomycin (an inhibitor of gene transcription), cycloheximide (an inhibitor of protein synthesis) and spironolactone (a mineralocorticoid receptor (MR) antagonist). Markos et al. [8] demonstrated that ALDO causes a rapid nongenomic increase in NHE1 activity in M-1 cortical collecting duct cells via the PKC/MAPK pathway; they also found that this effect is independent of MR. Gekle et al. [30] also verified a rapid activation of NHE1 in MDCK cells after approximately 5 min of exposure to ALDO.

The present results indicate that the lowest dose of ALDO (10^{-12} M) increases the speed of H^+ extrusion and, therefore, stimulates the NHE1 exchanger; on the other hand, the higher dose of ALDO (10^{-6} M) decreases the speed of H^+ extrusion and, therefore, inhibits this transporter, showing once again the dose-dependent biphasic effect of ALDO in NHE1. The receptor involved in the rapid responses of ALDO in non-polarized and polarized cells, including renal epithelial cells, is still unknown. However, in an attempt to identify the receptor of the nongenomic effect of ALDO on NHE1 in the S3 segment, we studied the action of spironolactone (a MR antagonist) and RU 486 (a GR antagonist) on the pHirr and $[Ca^{2+}]_i$, in the presence and absence of ALDO. Spironolactone alone did not alter the pHirr or the $[Ca^{2+}]_i$ and failed to prevent the short-term effects of ALDO (10^{-12} and 10^{-6} M) on these parameters. Consistent with our results, some studies showed nongenomic spironolactone–insensitive effects of aldosterone in vascular smooth muscle cells [33], in renal epithelial cells [7,8,34,35], in the glomerular microcirculation [36] and in medullary thick ascending limb [10]; whereas the present results demonstrated this effect in proximal tubule. RU 486 alone decreased the pHirr and $[Ca^{2+}]_i$, prevented the stimulatory effect of ALDO (10^{-12} M) on both parameters, maintained the inhibitory effect of ALDO (10^{-6} M) on pHirr and reversed the stimulatory effect of ALDO (10^{-6} M) on $[Ca^{2+}]_i$ to an inhibitory effect. Considering these results and the fact that the nongenomic ALDO action on the proximal NHE1 and NHE3 isoforms is sensitive to GR antagonism [2,5] and that GR is much more abundant than the MR in the proximal tubule [37], it is plausible to suggest that GR participates in the nongenomic effect of ALDO in the present experiments.Our present results show that ANP alone has no effect on the Na^+/H^+ exchanger; this behavior is in agreement with our data showing that, in MDCK cells, ANP (10^{-6} M) does not affect this exchanger [19]. However, in rat aortic smooth muscle (RASM) cells, 10^{-10} M ANP activates the Na^+/H^+ exchanger and 10^{-7} M ANP inhibits it [38]; in addition, in the ocular nonpigmented ciliary epithelium (NPE) cells, 10^{-7} M ANP has an inhibitory effect on the Na^+/H^+ exchanger activity [39].

A possible explanation for these different results would be that the direct effect of ANP on Na^+/H^+ exchanger depends on the cell type.

However, the present study is the first demonstration, to our knowledge, that ANP inhibits the nongenomic biphasic effect of ALDO on NHE1 in proximal S3 segment of rat. This action was demonstrated by prevention of the change in pHirr when the S3 segment was superfused with ANP and ALDO (10^{-12} or 10^{-6} M). Therefore, our data are in accordance with the studies in the rat proximal convoluted tubule showing that ANP inhibits the bicarbonate [18] and sodium [16,17] reabsorption stimulated by low doses of ANG II and with the experiments in MDCK cells demonstrating that ANP abolishes the stimulatory and inhibitory effects of ANG II [19] or AVP [20], despite the lack of effect of ANP alone on proximal convoluted tubule and MDCK cells.

To obtain more information about the nongenomic mechanism of interaction of ANP and ALDO on the modulation of pHi in the S3 segment, we also studied the effects of ANP with ALDO (2 min preincubation) on the regulation of $[Ca^{2+}]_i$. The present data indicate that the baseline $[Ca^{2+}]_i$ was 104 ± 3 nM (15) and that after addition of ALDO (10^{-12} or 10^{-6} M) to the bath, there was a rapid (approximately 0.4 min) dose-dependent increase of the $[Ca^{2+}]_i$. Gekle et al. [7] demonstrated that the elevation of $[Ca^{2+}]_i$ participates in the fast activation of ALDO on the Na^+/H^+ exchanger in renal epithelial cells, and our current results indicate that the rapid and biphasic aldosterone-induced effect on Na^+/H^+ exchanger is probably associated with the increase of $[Ca^{2+}]_i$.

Some studies [19,40] have found that the NHE1 exchanger has two calmodulin binding sites at the cytoplasmic regulatory domain that modulate its activity. A high-affinity site, which is tonically inhibitory, binds to low Ca^{2+}/calmodulin levels, thus suppressing the inhibition (i.e., stimulating the exchanger at low Ca^{2+}/calmodulin levels). A low affinity site, however, binds to Ca^{2+} and calmodulin only at high concentrations and, under these conditions, inhibits the exchanger activity. More recently, we modified amino acids in these two binding sites of NHE1 by site-directed mutagenesis and obtain data that reinforce this idea [41]. This behavior is compatible with our present findings, indicating stimulation of the NHE1 exchanger by increases of $[Ca^{2+}]_i$ in the lower range (at 10^{-12} M ALDO) and inhibition of this exchanger at high $[Ca^{2+}]_i$ levels (at 10^{-6} M ALDO). The results using RU 486 also agree with this NHE1 regulation by alterations in the $[Ca^{2+}]_i$, because this GR antagonist alone or with ALDO (10^{-12} or 10^{-6} M) decreased the $[Ca^{2+}]_i$, abolishing the stimulatory effect of ALDO (10^{-12} M) and maintaining the inhibitory effect of ALDO (10^{-6} M) on the pHirr.

Several actions of ANP depend on its interaction with type B receptors, coupled to the activation of guanylyl cyclase in the membrane that leads to increased levels of cGMP from GTP [14,42]. The elevation of cGMP may inhibit the activity of phospholipase C or stimulate the Ca^{2+}-ATPase of the sarcoplasmic reticulum, with the consequent reduction of $[Ca^{2+}]_i$ [43]. Our present results show that the addition of ANP alone to the bath decreases the $[Ca^{2+}]_i$ to approximately 44% of the control value. In the presence of ANP with ALDO (10^{-12} or 10^{-6} M), there is a dose-dependent recovery of $[Ca^{2+}]_i$, but the $[Ca^{2+}]_i$ does not reach ALDO (10^{-12} or 10^{-6} M) alone values. These findings are consistent with our results concerning the effect of this hormone on the pHirr. ANP alone does not affect the pHirr because it only causes a moderate decrease in $[Ca^{2+}]_i$. On the other hand, ANP impairs both the stimulatory and inhibitory effects of ALDO on the pHirr because it impairs the increase in $[Ca^{2+}]_i$ in response to ALDO, thus modulating the nongenomic cellular action of ALDO. The effect of this hormonal interaction on the pHirr and on $[Ca^{2+}]_i$ is similar to the rapid effect we observed with ANP with ANG II [19] or AVP [20] in MDCK cells.

In the present experiments, BAPTA, an intracellular calcium chelator, was used to confirm the effects of the decrease on $[Ca^{2+}]_i$ in NHE1 activity. BAPTA (5×10^{-5} M) alone or with ALDO (10^{-12} or 10^{-6} M) decreased the $[Ca^{2+}]_i$ by approximately 50% and blocked both the stimulatory and inhibitory effects of ALDO on NHE1 activity. These results are in accordance with a recent study, also in the S3 segment, wherein BAPTA prevented the increase of $[Ca^{2+}]_i$ and the H^+-ATPase activity in response to ALDO [27].

5. Conclusions

Our current studies in the isolated proximal straight tubule suggest a role for $[Ca^{2+}]_i$ in regulating the process of pHi recovery after the acid load induced by NH_4Cl, which is mediated by the basolateral NHE1 exchanger and stimulated/impaired by ALDO via a nongenomic pathway. The results are compatible with stimulation of the NHE1 exchanger by increases in $[Ca^{2+}]_i$ in the lower range (at 10^{-12} M ALDO) and inhibition at high $[Ca^{2+}]_i$ levels (at 10^{-6} M ALDO). This finding is also compatible with the identification of two sites on the COOH terminus of the NHE1 exchanger: one that stimulates the exchanger activity at low $[Ca^{2+}]_i$ levels, and one that inhibits this activity at high $[Ca^{2+}]_i$. ANP and BAPTA decrease $[Ca^{2+}]_i$ to approximately 45–50% of the control value and do not affect the pHi recovery, but these compounds impair the increase in $[Ca^{2+}]_i$ and block both the stimulatory and inhibitory effects of ALDO on this process. RU 486, but not spironolactone, interferes with the increase of $[Ca^{2+}]_i$ and the nongenomic effect of ALDO on NHE1, suggesting that the GR could participate in this mechanism. Although we have shown evidence for the importance of $[Ca^{2+}]_i$, it is possible that signaling mechanisms besides $[Ca^{2+}]_i$ contribute to the nongenomic action of ALDO on NHE1 exchanger. The observed nongenomic hormonal interaction in the proximal straight tubule (a less studied proximal tubular portion) may represent a mechanism for the rapid and physiologically relevant regulation in conditions of volume depletion or expansion in the intact animal.

Disclosure

No conflicts of interest, financial or otherwise, are declared by the authors.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Pesquisas (CNPq).

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