ANP impairs the dose-dependent stimulatory effect of ANG II or AVP on $\text{H}^+\text{-ATPase}$ subcellular vesicle trafficking *

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Received 27 May 2013; revised 22 June 2013; accepted 5 July 2013

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ABSTRACT

The effect of angiotensin II (ANG II) or arginine vasopressin (AVP) alone or plus atrial natriuretic peptide (ANP) on $\text{H}^+\text{-ATPase}$ subcellular vesicle trafficking was investigated in MDCK cells following intracellular pH (pHi) acidification by exposure to 20 mM NH$_4$Cl for 2 min in a Na$^+$-free solution containing Schering 28080, conditions under which H$^+$-ATPase is the only cell mechanism for pHi recovery. Using the acridine orange fluorescent probe (5 μM) and confocal microscopy, the vesicle movement was quantified by determining, for each experimental group, the mean slope of the line indicating the changes in apical/basolateral fluorescence density ratio over time during the first 5.30 min of the pHi recovery period. Under the control conditions, the mean slope was $0.079 \pm 0.0033 \text{ min}^{-1}$ (14) and it increased significantly with ANG II $[10^{-12} \text{ and } 10^{-7} \text{ M}]$, respectively to $0.322 \pm 0.038 \text{ min}^{-1}$ (13) and $0.578 \pm 0.061 \text{ min}^{-1}$ (12) or AVP $[10^{-12} \text{ and } 10^{-6} \text{ M}]$, respectively to $0.301 \pm 0.018 \text{ min}^{-1}$ (12) and $0.687 \pm 0.049 \text{ min}^{-1}$ (11). However, in presence of ANP ($10^{-8} \text{ M}$, decreases cytosolic free calcium), dimethyl-BAPTA/AM ($5 \times 10^{-5} \text{ M}$, chelates intracellular calcium) or colchicine ($10^{-5} \text{ M}$, 2-h preincubation; inhibits microtubule-dependent vesicular trafficking) alone or plus ANG II or AVP the

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Keywords: $\text{H}^+\text{-ATPase}$ Vesicle Trafficking; ANP; ANG II; AVP

1. INTRODUCTION

The effect of angiotensin II (ANG II) on $\text{H}^+\text{-ATPase}$ activity is controversial. In proximal [1] and distal [2] rat kidney tubules and in intercalated cells of connecting tubule [3], cortical [3,4] and medullary [5] collecting duct ANG II ($10^{-12} - 10^{-9} \text{ M}$) stimulates $\text{H}^+\text{-ATPase}$. However, in the rat cortical [6] and outer medullary [7] collecting duct, ANG II ($10^{-10} - 10^{-5} \text{ M}$) causes a dose-dependent decrease in $\text{H}^+\text{-ATPase}$. In addition, our studies in MDCK cells, a permanent cell line originated from the renal collecting duct, indicate that after intracellular pH (pHi) acidification using an NH$_4$Cl pulse, ANG II ($10^{-12}$, $10^{-9}$ or $10^{-7} \text{ M}$) stimulates $\text{H}^+\text{-ATPase}$. However, in the rat cortical [6] and outer medullary [7] collecting duct, ANG II ($10^{-10} - 10^{-5} \text{ M}$) causes a dose-dependent decrease in $\text{H}^+\text{-ATPase}$ activity. In addition, our studies in MDCK cells, a permanent cell line originated from the renal collecting duct, indicate that after intracellular pH (pHi) acidification using an NH$_4$Cl pulse, ANG II ($10^{-12}$, $10^{-9}$ or $10^{-7} \text{ M}$) stimulates $\text{H}^+\text{-ATPase}$. However, in the rat cortical [6] and outer medullary [7] collecting duct, ANG II ($10^{-10} - 10^{-5} \text{ M}$) causes a dose-dependent decrease in $\text{H}^+\text{-ATPase}$ activity. In addition, our studies in MDCK cells, a permanent cell line originated from the renal collecting duct, indicate that after intracellular pH (pHi) acidification using an NH$_4$Cl pulse, ANG II ($10^{-12}$, $10^{-9}$ or $10^{-7} \text{ M}$) stimulates $\text{H}^+\text{-ATPase}$. However, in the rat cortical [6] and outer medullary [7] collecting duct, ANG II ($10^{-10} - 10^{-5} \text{ M}$) causes a dose-dependent decrease in $\text{H}^+\text{-ATPase}$ activity. In addition, our studies in MDCK cells, a permanent cell line originated from the renal collecting duct, indicate that after intracellular pH (pHi) acidification using an NH$_4$Cl pulse, ANG II ($10^{-12}$, $10^{-9}$ or $10^{-7} \text{ M}$) stimulates $\text{H}^+\text{-ATPase}$.
responsible for the increase in $[Ca^{2+}]_i$, blocking the stimulatory effect of ANG II on H+-ATPase [8].

The effect of arginine vasopressin (AVP) on H+-ATPase activity is unclear. In an in vivo microperfusion study, we demonstrated that in the late distal tubule of rat kidney, luminal AVP ($10^{-5}$ M) stimulates H+-ATPase via the activation of V1 receptors [9]. In principal and intercalated cell of rabbit cortical collecting duct, AVP increases cAMP accumulation [10]; however, it has been suggested that in this duct, luminal AVP ($10^{-5}$ M) impairs electrogenic H+ secretion [11] and in rat medullary thick ascending limb cells, AVP does not affect H+-ATPase directly [12]. In addition, our data with MDCK cells suggest that the increase in $[Ca^{2+}]_i$ in response to AVP and blocks the stimulatory effect of AVP on H+-ATPase [13].

Moreover, it is known that: 1) acute cellular acidification stimulates exocytosis and elicits a rapid increase in proton secretion that is mediated by an H+-ATPase [14], 2) an increase in $[Ca^{2+}]_i$ might reflect a physiological mechanism to stimulate H+-ATPase-mediated protein export under acidic conditions [15,16], 3) cAMP stimulates V-ATPase accumulation, microvillar elongation, and proton extrusion in kidney collecting duct A-intercalated cells [17] and 4) vesicle trafficking and exocytosis play a role in the regulation of H+ transport in MDCK cells [18,19].

Based on these findings, in the present study we investigated the effect of ANG II ($10^{-12}$ and $10^{-7}$ M) or AVP ($10^{-12}$ and $10^{-6}$ M) alone or plus ANP ($10^{-6}$ M) on the subcellular acidic vesicle trafficking in MDCK cells following intracellular acidification using NH4Cl. The experiments were performed in a Na+-free solution containing Schering 28080 (specifically inhibits H7/K-ATPase), experimental conditions under which H+-ATPase secretion [11] and in rat medullary thick ascending limb cells, AVP does not affect H+-ATPase directly [12]. In addition, our data with MDCK cells suggest that the increase in $[Ca^{2+}]_i$ in response to AVP and blocks the stimulatory effect of AVP on H+-ATPase [13].

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AOH$^+$ in the interior, where it accumulates. Therefore, as the vesicle becomes more acidic, the AOH$^+$ level becomes higher, decreasing the fluorescence; however, agents that stimulate the proton pumps make the vesicle less acidic and thus more fluorescent [24,26].

In the present work the H$^+$-ATPase activity was assessed as the rate of increase in fluorescence occurring during the pH$_i$ recovery period after acid loading, in a Na$^+$-free solution containing Schering 28080. In the control conditions, it was noted that during the pH$_i$ recovery period, the largest concentration of vesicles is around the nucleus and/or at the basolateral side of the cells. However, in presence of ANG II or AVP, as pH$_i$ recovery proceeds, the density of vesicles at the apical pole of the cells increases, suggesting their transfer toward this pole (Figures 1(a) and 2(a)).

To detect the movement of the cytoplasmic vesicles inside the cells during the pH$_i$ recovery period, the acridine orange fluorescence was measured [27] for a total of 10 min and a $z$ sequence, at stepwise depths of 1.4 μm, was recorded every 82 s. The $z$ sequence started at the apical cell surface (0 μm) and ended at the basolateral surface (9.8 μm). The Figures 1 and 2 demonstrate the sequence of fluorescence images taken over time and space.

Figure 1. Confocal microscopy images of MDCK cells preincubated with acridine orange. Effect of ANG II (10$^{-12}$ and 10$^{-7}$ M) or/and ANP (10$^{-8}$ M) on the movement of acidic vesicles within the cells during the pH$_i$ recovery period via H$^+$-ATPase after the acid loading induced by NH$_4$Cl. The vesicular trafficking was followed by measuring the fluorescence density from the basolateral (lower) to the apical (upper) areas. Under the control conditions, during the entire period of pH$_i$ recovery the largest concentration of fluorescence was observed at the basolateral side of the cells. In presence of ANG II alone (a), as the pH$_i$ recovery progressed, the fluorescence density at the apical pole of the cells increased in a dose-dependent manner, suggesting the transfer of the acidic vesicles toward this pole. With ANP alone or plus ANG II (b), as the pH$_i$ recovery proceeded, the largest concentration of fluorescence was observed at the basolateral side of the cells, suggesting that the movement of the vesicles toward the apical pole did not occur. Objective 63×, pinhole 347, fluorescence density (in green).
Figure 2. Confocal microscopy images of MDCK cells preincubated with acridine orange. Effect of AVP \(10^{-12}\) and \(10^{-6}\) M or/and ANP \(10^{-6}\) M on the movement of acidic vesicles within the cells during the pH recovery period via H⁺-ATPase after the acid loading induced by NH₄Cl. The vesicular trafficking was followed by measuring the fluorescence density from the basolateral (lower) to the apical (upper) areas. In the presence of AVP alone (a), as the pH recovery progressed, the fluorescence density at the apical pole of the cells increased in a dose-dependent manner, suggesting the transfer of the acidic vesicles toward this pole. With AVP plus ANP (b), as the pH recovery proceeded, the largest concentration of fluorescence was not observed at the apical side of the cells, indicating that the movement of the vesicles toward this pole did not occur. Objective 63×, pinhole 347, fluorescence density (in yellow).

indicate the z-axis depth. The images were stored on a CD and were analyzed using the Adobe Photoshop 6.0 image program. The movement of the vesicles was determined in 10 cells by measuring the fluorescence density of the cytoplasm from the basolateral to the apical areas (outside the cell nucleus). This process was quantified by determining the time course of the apical to basolateral cytoplasmic fluorescence density ratio [25]. The experiments were performed under control conditions or in the presence of ANG II \(10^{-12}\) and \(10^{-7}\) M) or AVP \(10^{-12}\) and \(10^{-6}\) M) and/or ANP \(10^{-6}\) M), BAPTA (\(5 \times 10^{-5}\) M) or colchicine (\(10^{-5}\) M, 2-h preincubation).

2.3. Solutions and Reagents

The osmolality of the solutions was approximately 300 mOsmol/Kg H₂O, which is the osmolality of the culture medium. ANP (28-aminacid) was purchased from Bachem Fine Chemicals (New Haven, CT, USA) and BAPTA was from Molecular Probes (Eugene, OR, USA). The ANG II (1046 molecular weight), AVP (molecular weight 1.084) and all other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO, USA).

2.4. Statistics

The results are presented as the means ± SEM; (n) is the number of experiments. The data were analyzed statistically by analysis of variance followed by Bonferroni’s contrast test. Differences were considered significant if p < 0.05.

The Biomedical Sciences Institute, University of São Paulo, Ethical Committee for Animal Research (CEEA) approved this study.

3. RESULTS

Figure 3 shows the fluorescent cytoplasmic vesicles in an MDCK cell preincubated under the preliminary experimental conditions, i.e., in the Na⁺-free solution containing Schering 28080 and in the absence of acid loading. The data indicate that acridine orange is taken up rapidly by the cell, concentrates in cytoplasmic vesicles and is not lost during the initial 12 min of the experiment (upper) and that ANG II \(10^{-12}\) M) increases the number of vesicles that can be detected in the cytoplasm (lower).

Figures 1 and 2 demonstrate the movement of the fluorescent vesicles inside the cells during the pH recovery period following the NH₄Cl loading. As the pH recovery proceeded, under the control conditions the largest concentration of fluorescence was observed at the basolateral side of the cells over time, and with ANG II \(10^{-12}\) and \(10^{-7}\) M) the fluorescence density at the apical pole of the cells exhibited a dose-dependent increase.
Figure 3. Fluorescent cytoplasmic vesicles in an MDCK cell preincubated with acridine orange in a Na⁺-free solution containing Schering 28080 in the absence of acid loading, under the control conditions (upper). The presence of ANG II (10⁻¹² and 10⁻⁷ M) caused an increase in the number of vesicles that can be detected in the cytosol (lower). N = nuclei. C = cytoplasm. Bar ≈ 0.5 μm.

with time (Figure 1(a)). However, an increase in the fluorescence density at the apical pole was not observed with ANP alone or in combination with ANG II (10⁻¹² and 10⁻⁷ M) (Figure 1(b)). As the pH recovery progressed in the presence of AVP (10⁻¹² and 10⁻⁹ M) the fluorescence density at the apical pole of the cells exhibited a dose-dependent increase with time (Figure 2(a)); nevertheless, it did not increase in the presence of AVP (10⁻¹² and 10⁻⁶ M) plus ANP (Figure 2(b)).

In addition, the results also indicate that, similar to what occurs with ANP, in the presence of BAPTA or colchicine alone or plus ANG II or AVP, as the pH recovery proceeded, the largest concentration of fluorescence was observed at the basolateral side of the cells, suggesting that the movement of the vesicles toward the apical pole was inhibited (Figures not shown).

Figure 4 ((a) upper and (b) upper) shows that, as the pH recovery proceeded, under the control conditions the apical/basolateral fluorescence density ratio (FDap/FDb) remained almost constant over time. Already with ANG II (10⁻¹² and 10⁻⁷ M) the FDap/FDb increased markedly, particularly at 10⁻⁷ M ANG II (Figure 4(a) upper); however, in the presence of ANG II (10⁻⁷ M) in combination with ANP, BAPTA or colchicine the (FDap/FDb) demonstrated only minor changes over time (Figure 4(a) lower). In the presence of AVP (10⁻¹² and 10⁻⁶ M), the FDap/FDb exhibited a dose-dependent increase with time (Figure 4(b) upper); nevertheless, with AVP (10⁻⁶ M) plus ANP, BAPTA or colchicine the FDap/FDb did not change significantly with time (Figure 4(b) lower). The mean slopes (changes in FDap/FDb over time) of the lines shown in Figure 4 were calculated during the first 5.30 min of the pH recovery period. Table 1 indicates that under the control conditions the mean slope was 0.079 ± 0.0033 min⁻¹ (n = 14), and that it increased significantly in the presence of ANG II, in a dose-dependent manner. However, this increase was not observed with ANP, BAPTA or colchicine alone or in combination with ANG II. Table 1 also demonstrates that in the presence of AVP the mean slopes increased markedly, in a dose-dependent manner. Yet, similar to ANG II, this increase was not observed with AVP plus ANP, BAPTA or colchicine.

4. DISCUSSION

The aim of this study was to clarify the influence of ANG II or AVP alone or in combination with ANP or other agents, on H⁺-ATPase subcellular vesicle trafficking after intracellular acidification by NH₄Cl, in MDCK cells strain I and subtype C11. Because two-thirds of these cells exhibited a peanut lectin binding capacity [23], they resemble intercalated cells in the renal collecting duct and are presumably the cells in which the H⁺-ATPase under investigation is localized. Supporting this proposal, the two Na⁺-independent proton secretion mechanisms found in these cells, the H⁺/K⁺-ATPase and the vacuolar H⁺-ATPase [28,29], are similar to the mechanisms found in the intercalated cells of the mammalian collecting duct. The experiments were performed in an Na⁺-free solution (to inhibit the Na⁺/H⁺ exchanger) and in the presence of Schering 28080 (to inhibit the H⁺/K⁺-ATPase), experimental conditions under which H⁺-ATPase provides the only mechanism for pH recovery in these cells [13], which is inhibited by concanamycin (a specific inhibitor of the vacuolar H⁺-ATPase) [29].
Figure 4. Mean time course of the apical (0 μm) to basolateral (9.4 μm) fluorescence density ratio (F_Dap/F_Dbl) in MDCK cells loaded with acridine orange during the pHi recovery via H^+-ATPase after the acid loading. Under the control conditions, the F_Dap/F_Dbl did not change significantly over time. However, in the presence of ANG II (10^{-12} and 10^{-7} M) (a) or AVP (10^{-12} and 10^{-6} M) (b), the F_Dap/F_Dbl increased markedly in a dose-dependent manner. But, with ANG II (10^{-7} M) or AVP (10^{-6} M) (a) or (b), respectively) plus ANP, BAPTA or colchicine, the F_Dap/F_Dbl exhibited only minor changes over time, indicating that the movement of the vesicles was inhibited. N = 10 cells.

Table 1. Effect of ANG II or AVP alone or plus ANP, BAPTA or colchicine on changes of apical/basolateral fluorescence density ratio over time, on MDCK cells loaded with acridine orange. The values were calculated during the first 5.30 min of pHi recovery after the pHi acidification by NH_4Cl in a Na^-free solution containing Schering 28080.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>min⁻¹</th>
<th>Experimental groups</th>
<th>min⁻¹</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.079 ± 0.0033 (14)</td>
<td>ANG II (10^{-12} M)</td>
<td>0.322 ± 0.038* (13)</td>
</tr>
<tr>
<td>ANG II (10^{-7} M)</td>
<td>0.578 ± 0.061* (12)</td>
<td>ANG II (10^{-12} M)</td>
<td>0.301 ± 0.018* (12)</td>
</tr>
<tr>
<td>ANP (10^{-6} M)</td>
<td>0.058 ± 0.015 (10)</td>
<td>AVP (10^{-12} M)</td>
<td>0.687 ± 0.049* (11)</td>
</tr>
<tr>
<td>ANP + ANG II (10^{-12} M)</td>
<td>0.050 ± 0.0094* (11)</td>
<td>ANP + AVP (10^{-12} M)</td>
<td>0.050 ± 0.0098* (12)</td>
</tr>
<tr>
<td>ANP + ANG II (10^{-7} M)</td>
<td>0.048 ± 0.0085* (13)</td>
<td>ANP + AVP (10^{-6} M)</td>
<td>0.049 ± 0.0081* (11)</td>
</tr>
<tr>
<td>BAPTA (5 x 10^{-7} M)</td>
<td>0.015 ± 0.013 (12)</td>
<td>BAPTA + AVP (10^{-12} M)</td>
<td>0.014 ± 0.0083* (12)</td>
</tr>
<tr>
<td>BAPTA + ANG II (10^{-12} M)</td>
<td>0.015 ± 0.0006 (12)</td>
<td>BAPTA + AVP (10^{-6} M)</td>
<td>0.050 ± 0.0038* (11)</td>
</tr>
<tr>
<td>Colchicine (10^{-6} M)</td>
<td>0.051 ± 0.018 (10)</td>
<td>Colchicine + AVP (10^{-12} M)</td>
<td>0.050 ± 0.0015* (12)</td>
</tr>
<tr>
<td>Colchicine + ANG II (10^{-12} M)</td>
<td>0.049 ± 0.0011* (11)</td>
<td>Colchicine + AVP (10^{-6} M)</td>
<td>0.049 ± 0.0091* (12)</td>
</tr>
<tr>
<td>Colchicine + ANG II (10^{-7} M)</td>
<td>0.048 ± 0.0088* (13)</td>
<td>Colchicine + AVP (10^{-6} M)</td>
<td>0.049 ± 0.0091* (12)</td>
</tr>
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</table>

Values are means ± SE (number of experiments). ANG II = angiotensin II; AVP = arginine vasopressin; ANP = atrial natriuretic peptide; BAPTA = dimethyl-BAPTA/AM (an intracellular calcium chelator); Colchicine = an inhibitor of microtubule-dependent vesicular trafficking and Schering 28080 = a specific inhibitor of H^+/K^+-ATPase. *p < 0.01 vs. Control; **p < 0.05 vs. BAPTA alone; †p < 0.01 vs. ANG II (10^{-12} M) alone; ‡p < 0.01 vs. ANG II (10^{-7} M) alone; §p < 0.01 vs. AVP (10^{-12} M) alone and ‡p < 0.01 vs. AVP (10^{-6} M) alone.
Under the preliminary experimental conditions in the absence of acid loading, our data indicate that the acridine orange was taken up rapidly by the cell, concentrated in the cytoplasmic vesicles and is not lost during the initial 12 min. Thus, these data indicate that the time that the acridine orange remains inside the cell is greater than the time required to detect the movement of the acidic cytoplasmic vesicles inside the cells during the pHi recovery period following the NH₄Cl loading (10 min).

4.1. ANG II

The present results show that, under the preliminary experimental conditions in the absence of acid loading, ANG II (10⁻¹² M) increases the number of vesicles that can be detected in the cytosol, since ANG II stimulates the proton pumps making the vesicles less acidic and thus more fluorescent [24,26]. During the pHi recovery period following pH increment, our results demonstrate a dose-dependent stimulatory effect of ANG II (10⁻¹² or 10⁻⁹ M) on H⁺-ATPase subcellular vesicle trafficking. These results are compatible with: 1) studies in MDCK cells showing that ANG II (10⁻¹² and 10⁻⁹ M) stimulated the pH recovery rate via H⁺-ATPase and the [Ca²⁺]i increase, in a dose-dependent manner [8]; 2) results in isolated rat proximal tubule fragments [1] and cells in culture [30], indicating that ANG II (10⁻⁹ M) stimulates proton extrusion via H⁺-ATPase by a process involving the membrane insertion of vesicles; 3) data from the cortical collecting duct of mice showing that the addition of ANG II to type A intercalated cells increases the ratio of apical plasma membrane H⁺-ATPase to cytoplasmic H⁺-ATPase three-fold [31] and 4) more recent results in kidney intercalated cells suggesting that the subcellular localization of V-ATPase and its activity is coupled with the acid-base status via PKA and AMP-activated protein kinase [32].

4.2. AVP

The present data demonstrate that as the pHi recovery proceeds following the NH₄Cl pulse, AVP (10⁻¹² and 10⁻⁶ M), similar to ANG II, has a dose-dependent stimulatory effect on H⁺-ATPase subcellular vesicle trafficking. Our data are in accordance with: 1) our previous experiments in MDCK cells indicating that AVP (10⁻¹² and 10⁻⁶ M) has a dose-dependent stimulatory effect on the pH recovery rate via H⁺-ATPase with the activation of V₁ and V₂ receptors and the synergy of the Ca²⁺/PKC and cAMP/PKA pathways [13], 2) studies in principal and intercalated cell of rabbit cortical collecting system demonstrating that AVP increases cAMP accumulation [10] and induces a [Ca²⁺]i increase mediated by V₂ through a cAMP/PKA pathway [33] and 3) more recent data in collecting duct A-intercalated cells demonstrating that cAMP has profound effects on the distribution and function of the V-ATPase and on the stimulation of long membrane extensions and microvilli [17].

4.3. ANP plus ANG II or AVP

The current results indicate that ANP alone, or in combination with ANG II or AVP, does not affect the movement of the H⁺-ATPase subcellular vesicles. This behavior is in agreement with the studies showing that in the presence of ANP alone plus ANG II or AVP [13]: 1) the pH recovery rate via H⁺-ATPase did not differ significantly from the control condition and 2) the effect of ANP on [Ca²⁺]i. According these previous studies, ANP alone did not affect the speed of the pH recovery because it caused a decrease in [Ca²⁺]i of approximately 40% of the control value, which by itself does not impair cellular H⁺ secretion; however, ANP impaired the effect of ANG II or AVP on the speed of the pH recovery because it impairs the increase in [Ca²⁺]i in response to ANG II or AVP, thus modulating the activity of these hormones in the cell. In addition, our data are in accordance with studies suggesting that an increase in [Ca²⁺]i might stimulate H⁺-ATPase-mediated protein export under acidic conditions [15,16] and with studies showing that ANP inhibits the cAMP synthesis stimulated by AVP [34].

4.4. BAPTA plus ANG II or AVP

Similar to ANP, the shift in the fluorescence toward the apical pole of the cells does not occur with BAPTA alone or in combination with ANG II or AVP. Again, these data are consistent with previous findings concerning the action of BAPTA on the pH recovery via H⁺-ATPase and on the [Ca²⁺]i in presence of ANG II [5,8] or AVP [13]. That is, BAPTA alone does not modify the pH recovery once it decreases the [Ca²⁺]i to approximately 49% of the control value, which by itself does not impair H⁺ secretion [8,13]. Similar to ANP, BAPTA impairs the effect of ANG II or AVP on H⁺-ATPase subcellular vesicle trafficking and on the pH recovery because it impairs the stimulatory effects of these hormones on the increase in [Ca²⁺]i. However, our current results show that in the presence of BAPTA alone or combination with ANG II and AVP, the change in FDap/FDb increases exhibits a relatively small but significant difference compared to BAPTA alone (p < 0.05, Table 1). It is likely that this difference results from the significant increase in [Ca²⁺]i observed in the presence of BAPTA plus ANG II (10⁻⁷ M) (24% above the control value [8]) which might initiate the movement of the vesicles toward the apical pole. Our present data also indicate that a similar scenario occurs in the presence of BAPTA plus AVP (10⁻⁶ M) (Table 1), although in this
situation the increase in \([\text{Ca}^{2+}]\) observed was not significant (11% above the control value [13]).

4.5. Colchicine plus ANG II or AVP

Colchicine disrupts microtubular structures and has been implicated in the vesicular trafficking of \(\text{H}^+\)-ATPase along the nephron, especially in the translocation of intracellular vesicles [20]. Our data indicate that the fluorescence density at the apical pole did not increase in the presence of colchicine alone or plus ANG II or AVP. This behavior is consistent with data showing that in rat proximal tubular cells [1] and in intercalated cells from isolated mouse connecting tubules and cortical collecting ducts [3] the stimulation of \(\text{H}^+\)-ATPase by ANG II required an intact microtubular network since it was completely inhibited by colchicine. Therefore, our colchicine data strongly suggest that in MDCK cells, ANG II or AVP stimulate the microtubule-dependent vesicular trafficking of acidic vesicles during the Na" and K"-independent pH recovery period after the acid loading.

5. CONCLUSION

The present study in MDCK cells loaded with acridine orange indicates that, as the pH recovery via \(\text{H}^+\)-ATPase proceeded following the acid loading induced by an NH\(_4\)Cl pulse, a dose-dependent ANG II \((10^{-12} \text{ and } 10^{-7} \text{ M})\) or AVP \((10^{-12} \text{ and } 10^{-8} \text{ M})\) increase in fluorescence density at the cellular apical pole occurred, suggesting the transfer of acidic vesicles toward this pole and their possible incorporation into the apical membrane. This finding is consistent with our previous studies, also in MDCK cells, demonstrating a role for \([\text{Ca}^{2+}]\) in regulating the process of pH recovery mediated by \(\text{H}^+\)-ATPase and stimulated by ANG II \((10^{-12} \text{ and } 10^{-7} \text{ M})\) [8] or AVP \((10^{-12} \text{ and } 10^{-8} \text{ M})\) [13] in a dose-dependent manner. Our present data, indicating that ANP or BAPTA does not affect the \(\text{H}^+\)-ATPase subcellular vesicle trafficking, are in agreement with the results of our previous studies showing that ANP- and BAPTA-mediated decrease in \([\text{Ca}^{2+}]\) to approximately 40% and 49% of the control values, respectively, do not affect the pH recovery [8,13]. Furthermore, our current findings indicating that ANP or BAPTA impairs the effect of ANG II or AVP on \(\text{H}^+\)-ATPase subcellular vesicle trafficking also agree with our earlier findings showing that ANP or BAPTA inhibits the increase in \([\text{Ca}^{2+}]\) in response to ANG II or AVP, which blocks the stimulatory effect of these hormones on the pH recovery [8,13], respectively. Therefore, the present study indicates that the increase in \([\text{Ca}^{2+}]\), although not exclusive, plays a role in regulating the dose-dependent stimulatory effect of ANG II or AVP on \(\text{H}^+\)-ATPase subcellular vesicle trafficking. Whether \([\text{Ca}^{2+}]\) reduction represents an important direct mechanism for ANP impairs this dose-dependent stimulatory effect of ANG II or AVP, or is a side effect of other signaling pathways which will require additional studies. In addition, the current observation that the fluorescence density at the apical pole did not increase in the presence of colchicine plus ANG II or AVP strongly suggests that in MDCK cells, the microtubule-dependent vesicular trafficking of acidic vesicles is involved in the dose-dependent stimulatory effect of ANG II or AVP on \(\text{H}^+\)-ATPase subcellular vesicle trafficking impaired by ANP.

6. ACKNOWLEDGEMENTS

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Pesquisas (CNPq). The authors thank Dr. Antonio Carlos Cassola for the assistance with the confocal microscope.

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