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Effect of arginine vasopressin and ANP on intracellular pH and cytosolic free [Ca^{2+}] regulation in MDCK cells

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Effect of arginine vasopressin and ANP on intracellular pH and cytosolic free [Ca^{2+}] regulation in MDCK cells.

Background. The effects of arginine vasopressin (AVP) on intracellular pH (pH_i) are not clearly defined, and may vary with cell membrane surface and the hormonal doses being studied. Since cytosolic free calcium concentration ($[Ca^{2+}]_i$) has an important effect on cellular H^+ extrusion and it was shown that AVP increases $[Ca^{2+}]_i$ while atrial natriuretic peptide (ANP) reduces it, there may be some interaction between AVP and ANP during the regulation of pH_i.

Methods. The effects of AVP and/or ANP on pH_i and $[Ca^{2+}]_i$ were investigated in Madin-Darby canine kidney (MDCK) cells by the fluorescent probes BCECF-AM and Fluo 4-AM, respectively. The pH_i recovery rate was examined in the first two minutes following the acidification of pH_i with a NH_4Cl pulse.

Results. AVP (10^{-12} or 10^{-9} mol/L) stimulated the rate of the Na^+-dependent pH_i recovery, but AVP (10^{-6} mol/L) impaired it. At the apical membrane surface, specific V1 or V2 receptor antagonists did not alter the effects of AVP. At the basolateral membrane surface, the V1 antagonist returned both the stimulatory and inhibitory effects of AVP to control levels, and the V2 antagonist converted the inhibitory effect of AVP to a stimulatory effect. ANP (10^{-6} mol/L) or dimethyl-BAPTA-AM (50 μmol/L) impaired both the stimulatory and inhibitory effects of AVP. AVP increased $[Ca^{2+}]_i$ in a dose-dependent manner. ANP or dimethyl-BAPTA-AM decreased $[Ca^{2+}]_i$, and the subsequent addition of AVP caused only a partial recovery of $[Ca^{2+}]_i$.

Conclusions. The results are compatible with stimulation of the Na^+/H^+ exchanger by increases of $[Ca^{2+}]_i$ in the lower range (at 10^{-12} or 10^{-9} mol/L AVP, via basolateral V1 receptors) and inhibition at high $[Ca^{2+}]_i$ levels (at 10^{-6} mol/L AVP, via basolateral V1 and V2 receptors). ANP, by impairing the path causing the increase in $[Ca^{2+}]_i$, blocks both the stimulatory and inhibitory effects of AVP on Na^+-dependent pH_i recovery.

The nature of the mechanism underlying arginine vasopressin (AVP) action on intracellular pH (pH_i) regulation is not yet defined clearly. In mesangial cells, AVP stimulates the Na^+/H^+ exchanger [1, 2] as well as the Na^+-dependent or independent Cl^-/HCO_3^- exchangers [2]. In A6 cells (an amphibian distal nephron cell line), however, at either low (10^{-10} mol/L) or high (10^{-6} mol/L) concentrations AVP inhibits the basolateral Na^+/H^+ exchanger activity [3]. Studies in isolated perfused mouse medullary thick ascending limbs showed that AVP stimulates the basolateral transporters while it simultaneously inhibits the apical Na^+/H^+ exchanger [4].

In addition, most studies have detected AVP activity when applied at the basolateral surface, which is mediated mostly by V2 receptors via the adenylate cyclase/cAMP signaling system [5]. However, in recent years, V1 receptors have been detected both in apical and basolateral membrane domains, and have been shown to mediate AVP activity via phospholipase C/inositol 3,4,5-triphosphate (IP_3)/calcium signaling [5–7]. Previous data from our laboratory have shown that luminal AVP (10^{-9} mol/L) acts on H^+ secretion in both early and late distal tubules of rat kidney via activation of V1 receptors [8], whereas peritubular AVP (10^{-11} and 10^{-9} mol/L) acts to stimulate bicarbonate reabsorption in both of these segments via activation of V1 receptors, and that V2 receptors have a dose-dependent inhibitor effect, possibly mediated by cAMP (abstract; Musa-Aziz et al, *J Am Soc Nephrol* 11:7A, 2000). Thus, it is possible that the AVP response of Na^+/H^+ exchanger may vary with the cell type, cell membrane surface, and hormonal doses being studied.

Studies exploring the mechanisms that control H^+ secretion by acid-secreting epithelia have emphasized the importance of cytosolic free calcium concentration ($[Ca^{2+}]_i$) in this process [9]. Thus, it was shown that AVP increases $[Ca^{2+}]_i$ [10], while atrial natriuretic peptide (ANP) reduces it [11]. In addition, ANP has been shown to inhibit cAMP synthesis stimulated by AVP in rat renal papillary collecting tubule cells in culture [12]. Thus, since AVP stimulates Na^+/H^+ exchange [11], there may be some interaction between AVP and ANP in the regulation of pH_i.

The present work investigated the role of AVP (10^{-12}, 10^{-9}, and 10^{-6} mol/L) in the modulation of pH_i, as well

Key words: atrial natriuretic peptide, Na/H exchangers, V1 receptors, V2 receptors, acid-base balance, BATPA-AM.

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as the mechanism of interaction between AVP and ANP (10^{-6} mol/L) or dimethyl-1,2-bis (2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester (BATPA-AM; 50 μmol/L) in the modulation of pH_i and $[Ca^{2+}]_i$. Madin-Darby canine kidney (MDCK) cells, a permanent cell line that is among the best characterized renal epithelial cells, were used. Our previous study demonstrated that these cells possess a basolateral Na^+/H^+ exchanger accounting for the Na^+-dependent pH_i recovery [13]. The present investigation also measured the effect of the V1-receptor specific antagonist [(β-mercapto-β, β-cyclopentamethylene-propionyl[1], O-Me-Tyr[2], Arg[8]) vasopressin (MCMV); 10^{-5} mol/L] or the V2-receptor specific antagonist [(adamantaneacetyl[1], O-Et-D-Tyr[2], Val[4], aminobutyryl[6], Arg[8,9]) vasopressin; 10^{-5} mol/L], at either the apical or basolateral membrane surface on Na^+-dependent pH_i recovery.

Our studies indicate a role of $[Ca^{2+}]_i$ in regulating the process of pH_i recovery after the acid load induced by NH_4Cl, mediated by the basolateral Na^+/H^+ exchanger and stimulated/impaired by AVP. They are compatible with stimulation of the Na^+/H^+ exchanger by increases of $[Ca^{2+}]_i$ in the lower range (that is, 10^{-12} or 10^{-9} mol/L AVP; mediated by basolateral V1 receptors) and inhibition at high $[Ca^{2+}]_i$ levels (10^{-6} mol/L AVP; via activation of basolateral V1 receptors). They also are compatible with inhibition of the Na^+/H^+ exchanger at high cell cAMP levels (at 10^{-6} mol/L AVP, mediated by basolateral V2 receptors). ANP and dimethyl-BAPTA-AM, by causing a moderate decrease of $[Ca^{2+}]_i$, do not affect the pH_i recovery, but impair the path causing an increase in $[Ca^{2+}]_i$, thus blocking both the stimulatory and inhibitory effects of AVP in this process.

METHODS

Cell culture and fluorescent measurement of pH_i and $[Ca^{2+}]_i$ were done as we described previously [13].

Cell culture

Wild-type MDCK cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were used for all experiments (passages 60 to 66). Serial cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Cells were grown at 37°C, 95% humidified air and 5% carbon dioxide (pH 7.4) in a CO_2 incubator (Lab-Line Instruments, Melrose Park, IL, USA). The cells were harvested with trypsin in ethyleneglycol-bis (b-aminoethyl ether)-N, N′-tetraacetic acid (EGTA, 0.02%), and then seeded on sterile glass coverslips and incubated again for 72 hours in the same medium to become confluent.

Fluorescent measurement of pH_i

Intracellular pH was monitored using the fluorescent probe 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxy-methyl ester (BCECF-AM). Cells grown to confluence on glass coverslips were loaded with the dye by exposure for 20 minutes to 10 μmol/L BCECF-AM in solution 1 (Table 1). After BCECF-AM entered the cells it was rapidly converted to the anionic free acid form by intracellular esterases. Following the loading period, the glass coverslips were rinsed with the control solution to remove the BCECF-containing solution and 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 40 cells. The coverslips remained in a fixed position, so that the same cells were studied throughout the experiment. Bathing solutions were rapidly exchanged without disturbing the position of the coverslips. All experiments were performed at 37°C. The cells were alternately excited at 455 or 505 nm with a 150 W xenon lamp and the fluorescence emission was monitored at 530 nm by a photomultiplier-based fluorescence system (Georgia Instruments, PMT-400) at time intervals of five seconds. The 505/455 excitation ratio corresponds to a specific pH_i. At the end of each experiment, calibration of the BCECF signal was achieved by the high K^+-nigericin method [14], exposing the cells for 15 minutes to a K^+-HEPES buffer solution containing 10 μmol/L nigericin (solution 3; Table 1), at pH 6.5, 7.0, or 7.5.

Cell pH recovery

Cell pH recovery was examined following the acidification of pH_i with the NH_4Cl pulse technique [15] after a two-minute exposure to 20 mmol/L NH_4Cl (solution 4; Table 1) in the following situations: control (in the presence of external 145 mmol/L Na^+, solution 2; Table 1), absence of external Na^+ (solution 5; Table 1) or in the presence of AVP (10^{-12}, 10^{-9}, or 10^{-6} mol/L), V1 and/or V2 receptor antagonists (10^{-5} mol/L), ANP (10^{-6} mol/L), or dimethyl-BAPTA-AM (50 μmol/L). Since the rate of pH recovery depends on the value of cell pH achieved by the acid load [16], we used experiments in which these values were not significantly different between the studied groups (Table 2). In all of the experiments, the initial rate of pH_i recovery (dpH_i/dt, pH units per min) was calculated from the first two minutes of the recovery curve by linear regression analysis.

Fluorescent measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were monitored fluorometrically using the Ca^{2+}-sensitive probe fluo 4-AM. MDCK cells were grown to confluence on uncoated glass-bottomed microwells (Mat-Tek, Ashland, MA, USA) at a density

Table 1. Composition of solutions (in mmol/L)

	Solution 1	Solution 2 Control	Solution 3 K^+-HEPES	Solution 4 NH_4Cl	Solution 5 0 Na^+	Solution 6 Tyrode
NaCl	100	145	20	125		137
KCl	5	5	130	5	5	2.68
$MgCl_2$	1	1	1	1	1	0.49
$CaCl_2$	1	1.8	1	1.8	1.8	1.36
HEPES	50	30	5	30	30	
Na_2SO_4		1		1		
NaH_2PO_4		1		1		0.36
$NaHCO_3$						12
Glucose		10		10	10	5.6
NH_4Cl				20		
NMDG					145	
pH	7.2	7.4	7.5; 7.0; 6.5	8.0	7.4	7.4

Abbreviation is: NMDG, N-methyl-D-glucamine. HCl or NaOH was used in all Na^+-containing solutions to titrate to the appropriate pH, and HCl or KOH was used in the Na^+-free solution.

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

		Basal	Acid load	Recovery	
	N		pH_i		ΔpH_i/min
Control	62	7.17 ± 0.01	6.67 ± 0.03	7.14 ± 0.04	0.101 ± 0.005
Na^+ *0 mmol/L*	13	7.14 ± 0.02	6.61 ± 0.07	6.65 ± 0.07[b]	0.034 ± 0.009[a]
				+7.12 ± 0.03	+0.075 ± 0.013
AVP *10^{-12} mol/L*	14	7.17 ± 0.01	6.73 ± 0.06	7.15 ± 0.07	0.177 ± 0.012[a]
AVP *10^{-9} mol/L*	24	7.18 ± 0.03	6.65 ± 0.03	7.17 ± 0.02	0.131 ± 0.011[a]
AVP *10^{-6} mol/L*	10	7.16 ± 0.07	6.65 ± 0.04	6.84 ± 0.03[b]	0.033 ± 0.004[a]
Na^+ *0 mmol/L* + AVP *10^{-12} mol/L*	9	7.16 ± 0.01	6.73 ± 0.02	6.83 ± 0.03[b]	0.072 ± 0.013[c]
				+7.09 ± 0.07	+0.083 ± 0.010
Na^+ *0 mmol/L* + AVP *10^{-9} mol/L*	5	7.14 ± 0.01	6.64 ± 0.03	6.79 ± 0.06[b]	0.022 ± 0.011[d]
				+7.10 ± 0.04	+0.081 ± 0.009
ANP *10^{-6} mol/L*	10	7.15 ± 0.04	6.73 ± 0.03	7.13 ± 0.02	0.101 ± 0.009
ANP *10^{-6} mol/L* + AVP *10^{-12} mol/L*	5	7.17 ± 0.02	6.69 ± 0.04	7.12 ± 0.03	0.073 ± 0.009[c]
ANP *10^{-6} mol/L* + AVP *10^{-9} mol/L*	9	7.16 ± 0.03	6.71 ± 0.02	7.10 ± 0.02	0.094 ± 0.012[d]
ANP *10^{-6} mol/L* + AVP *10^{-6} mol/L*	10	7.14 ± 0.02	6.66 ± 0.02	7.02 ± 0.05[b]	0.104 ± 0.011[e]
BAPTA/AM *50 μmol/L*	8	7.11 ± 0.07	6.64 ± 0.03	7.01 ± 0.04	0.091 ± 0.007
BAPTA/AM *50 μmol/L* + AVP *10^{-12} mol/L*	6	7.13 ± 0.01	6.87 ± 0.03	7.11 ± 0.05	0.090 ± 0.018[c]
BAPTA/AM *50 μmol/L* + AVP *10^{-9} mol/L*	6	7.14 ± 0.04	6.71 ± 0.06	7.09 ± 0.02	0.086 ± 0.004[d]
BAPTA/AM *50 μmol/L* + AVP *10^{-6} mol/L*	8	7.15 ± 0.02	6.76 ± 0.03	7.10 ± 0.01[b]	0.102 ± 0.011[e]

Values are means ± SE; *N* is the number of observations. ΔpH_i/min is the pH_i recovery rate in the first 2 min; + with the return of Na^+ to the bathing solution.
[a] $P < 0.05$ vs. control ΔpH_i/min
[b] $P < 0.05$ vs. respective pH_i basal
[c] $P < 0.05$ vs. AVP 10^{-12} mol/L ΔpH_i/min
[d] $P < 0.05$ vs. AVP 10^{-9} mol/L ΔpH_i/min
[e] $P < 0.05$ vs. AVP 10^{-6} mol/L ΔpH_i/min

of 2.5×10^5 cells/mL. Twenty-four hours after plating, confluent cultures were loaded with 10 μmol/L fluo 4-AM at 37°C for 40 minutes and rinsed in Tyrode solution (solution 6; Table 1) containing 0.2% bovine serum albumin (pH 7.4). Cells were studied at room temperature and fluo 4 fluorescence intensity emitted above 520 nm was imaged by using ultraviolet laser excitation at 488 nm on a Zeiss LSM 510 real-time confocal microscope. The images were continuously acquired before and after the addition of experimental solutions, at time intervals of 10 seconds, for a total of 200 seconds. For each experiment the maximum fluorescent signal for 10 cells was averaged and then used for analysis. Transformation of the fluorescent signal to $[Ca^{2+}]_i$ was performed by calibration with ionomycin (30 μmol/L; maximum concentration) followed by EGTA (2.5 mmol/L; minimum concentration) according to the Grynkiewicz equation [17], using the dissociation constant of 345 nmol/L (according to the Molecular Probes catalog).

Solutions and reagents

The composition of the solutions utilized is described in Table 1. These solutions had an osmolality between 325 and 330 mOsm, which is the value found in the culture medium used for these cells. This osmolality was used to avoid changes when the cells were transferred

from the culture medium to the experimental solutions. Twenty-eight amino acid ANP was purchased from Bachem Fine Chemicals (New Haven, CT, USA) and fluo 4-AM, BCECF-AM and dimethyl-BATPA-AM from Molecular Probes (Eugene, OR, USA). AVP (molecular weight 1.084), V1-receptor specific antagonist [anti-V1; (β-Mercapto-β,β-cyclopentamethylene-propionyl[1], O-Me-Tyr[2],Arg[8]) vasopressin; (MCMV)], V2-receptor specific antagonist [anti-V2, (adamantaneacetyl[1], O-Et-D-Tyr[2], Val[4], aminobutyryl[6], Arg[8,9]) vasopressin], as well as all other applied chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Statistics

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

RESULTS

pH_i

In all experiments, the cell pH recovery was examined following the acidification of pH_i with the NH_4Cl pulse technique. Figure 1 shows three representative experiments. Cells were first bathed with the control solution (Table 1), exhibiting the basal pH_i. After a two-minute exposure to 20 mmol/L NH_4Cl, during which cell pH_i increased transiently, NH_4Cl removal caused a rapid acidification of pH_i as a result of the NH_3 efflux. In the presence of the control solution, the initial fall in pH_i was followed by a recovery of pH_i towards the basal value (Fig. 1A). The addition of AVP (10^{-12} mol/L) to the bath caused a significant increase of the velocity of pH_i recovery (Fig. 1B), whereas in the presence of AVP (10^{-6} mol/L), the velocity of pH_i recovery decreased significantly (Fig. 1C).

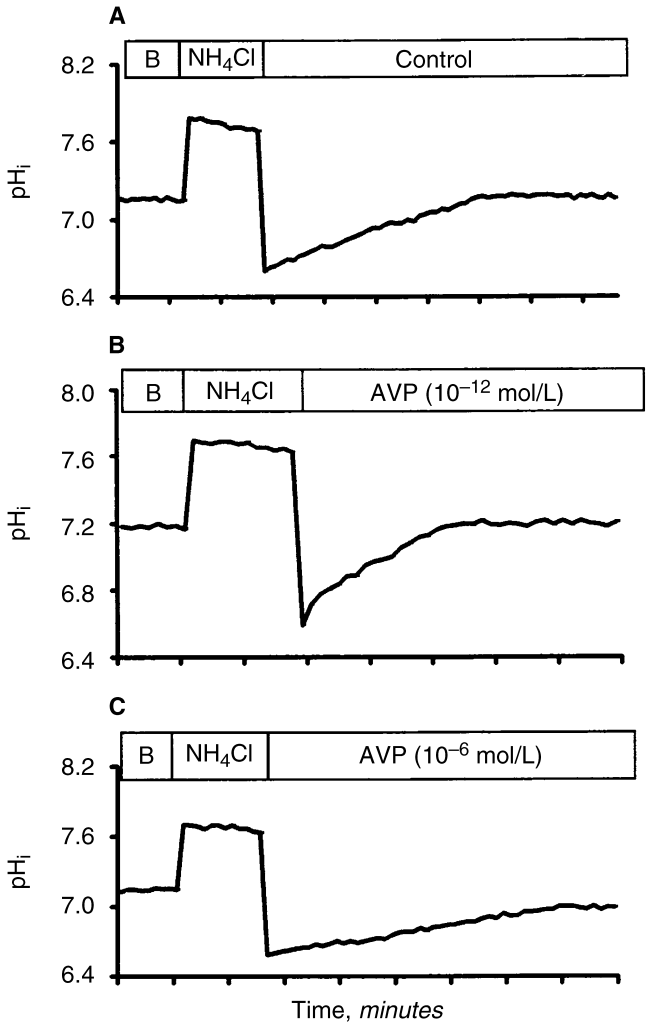

Fig. 1. Intracellular pH (pH_i) recovery after cellular acidification with the NH_4Cl pulse technique. (*A*) In the presence of control solution the initial fall in pH_i is followed by a recovery of pH_i towards the basal value. (*B*) The addition of 10^{-12} mol/L AVP to the bath causes a significant increase of the velocity of pH_i recovery. (*C*) The addition of AVP (10^{-6} mol/L) to the bath causes a significant decrease of the velocity of pH_i recovery. B, basal pH_i.

Table 2 summarizes the main values of pH_i responses found in all of the experimental groups. Our results found that MDCK cells in pH 7.4 HCO_3^-- free solution had a mean baseline pH_i of 7.15 ± 0.005 ($N = 199$).

Figure 2 indicates the effect of the absence of external Na^+ on the main pH_i recovery rate. In the control situation (that is, in the presence of external Na^+ 145 mmol/L), the main pH_i recovery rate in the first two minutes was 0.101 ± 0.005 pH units (U)/min ($N = 62$), and the final pH_i was not significantly different from the basal value (7.17 ± 0.01 vs. 7.14 ± 0.04; Table 2). In the absence of external Na^+, the pH_i recovery rate was reduced to 34% of the control value (and pH_i recovery was not complete; Table 2). This effect was reversed with the return of Na^+ to the bathing solution (and final pH_i was not significantly different from the basal value; Table 2), indicating that the pH_i recovery is mostly dependent on Na^+/H^+ exchange.

Figure 2 also shows that the addition of AVP (10^{-12} or 10^{-9} mol/L) to the bath causes a significant increase of the velocity of pH_i recovery (77 or 31% of the control value, respectively), and during both situations the final pH_i was not significantly different from the basal value (Table 2). However, the addition of AVP (10^{-6} mol/L) significantly decreased the velocity of pH_i recovery by 67% of the control value, and during this situation the pH_i recovery was not complete (Table 2). In the absence of external Na^+ both stimulatory effects of AVP were significantly inhibited and pH_i recovery was not complete (Table 2). With the return of Na^+ to the bathing solution both stimulatory effects of AVP subsequently were partly

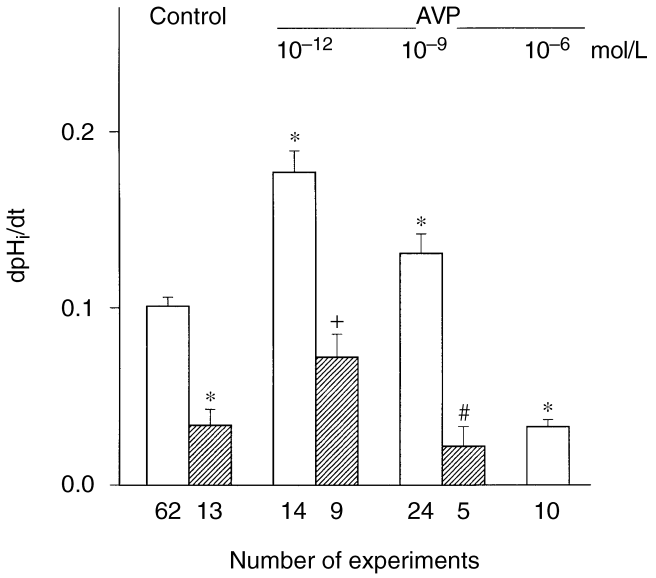

Fig. 2. Effect of arginine vasopressin (AVP; 10^{-12}, 10^{-9}, and 10^{-6} mol/L) on the initial rate of pH_i recovery following acute intracellular acidification in Madin-Darby canine kidney (MDCK) cells. The experiments were done in the presence of 145 mmol/L (□) or absence (▨) of extracellular Na^+. $*P < 0.05$ vs. control; $+P < 0.05$ vs. AVP (10^{-12} mol/L); $\#P < 0.05$ vs. AVP (10^{-9} mol/L).

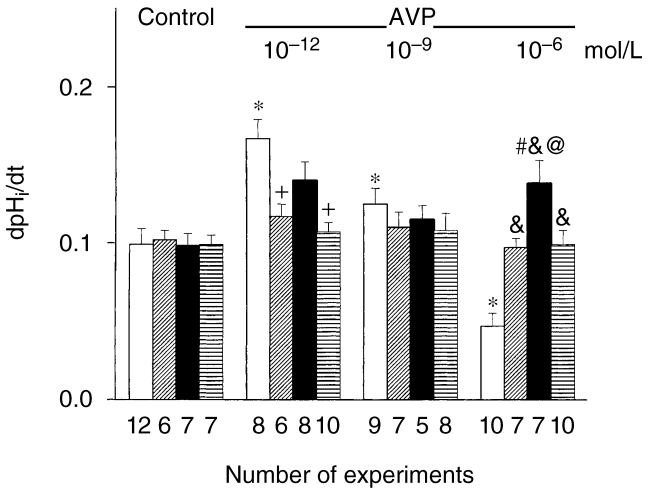

Fig. 3. Effect of V1 or V2 receptor antagonists (10^{-5} mol/L) alone or plus AVP (10^{-12}, 10^{-9}, or 10^{-6} mol/L) on the initial rate of pH_i recovery following acute intracellular acidification in MDCK cells. These experiments were done in cells growing on permeant filter supports in the presence of the agents at the basolateral membrane surface. Symbols are: (□) without antagonists; (▨) + anti-V1; (■) + anti-V2; (▤) anti-V1 + anti-V2. $*P < 0.05$ vs. control; $+P < 0.05$ vs. AVP (10^{-12} mol/L); $\#P < 0.05$ vs. anti-V2; $\&P < 0.05$ vs AVP (10^{-6} mol/L); $@P < 0.05$ vs. AVP (10^{-6} mol/L) + anti-V1.

recovered, indicating that they are mostly dependent on Na^+/H^+ exchange.

We performed a series of experiments in which MDCK cells were grown on permeant filter supports (Transwell 3.0 μm pore size, 12 mm diameter; Costar, Cambridge, MA, USA), making it possible to independently measure the effect of the V1 or V2-receptor antagonists on Na^+-dependent pH_i recovery at either the apical or basolateral membrane surface. Figure 3 summarizes the results found in the presence of the receptor antagonists at the basolateral membrane surface. In the control situation, the pH_i recovery rate in the first two minutes was 0.099 ± 0.01 ($N = 12$), a value not significantly different from 0.101 ± 0.005 pH U/min ($N = 62$), the control value found when the cells were grown on coverslips. In the presence of V1 and/or V2 receptor antagonists, the pH_i recovery rate was not significantly different from the control value. These data indicate that these antagonists have no intrinsic effects on pH_i responses. Figure 3 also shows that V1 or V1 plus V2 receptor antagonists return both the stimulatory and inhibitory effects of AVP to control levels. Figure 3 also indicates that the V2 receptor antagonist converts the inhibitory effect of AVP to a stimulatory effect. However, another series of experiments (data not shown) indicates that V1 or V2 receptor antagonists at the apical membrane surface did not affect either the stimulatory or inhibitory effects of AVP, showing that the inhibitors have no effect at the apical side and could not traverse the cells and membranes to the opposite side. Based on these data, we speculate that the V1 and V2 receptors responsible for the Na^+-dependent pH_i recovery observed in the present study are located on the basolateral membrane surface.

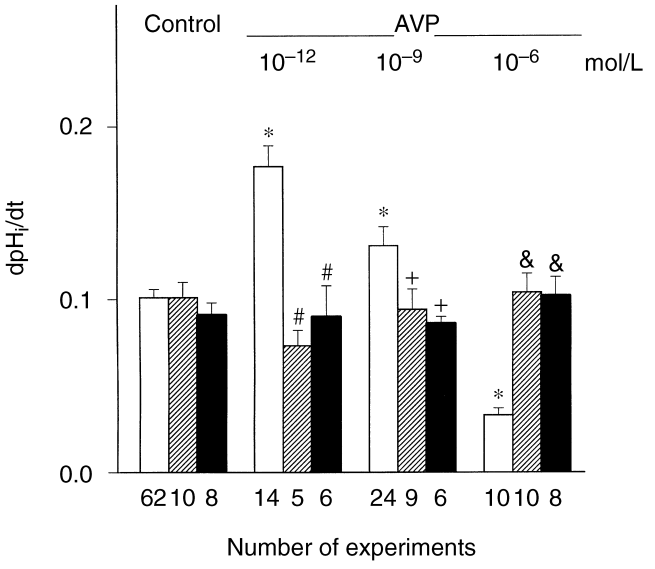

Fig. 4. Effects of arginine vasopressin (AVP; 10^{-12}, 10^{-9}, or 10^{-6} mol/L; □) and/or atrial natriuretic peptide (ANP, 10^{-6} mol/L; ▨), and/or dimethyl-BAPTA/AM (50μmol/L; ■) on the initial rate of pH_i recovery following acute intracellular acidification in MDCK cells. $*P < 0.05$ vs. control; $\#P < 0.05$ vs. AVP (10^{-12} mol/L); $+P < 0.05$ vs. AVP (10^{-9} mol/L); $\&P < 0.05$ vs. AVP (10^{-6} mol/L).

Figure 4 gives the effect of addition of ANP (10^{-6}

mol/L) alone or plus AVP (10^{-12}, 10^{-9}, or 10^{-6} mol/L) to the bath on the rate of pH_i recovery, again using MDCK cells on glass coverslips. With ANP alone the pH_i recovery rate was not significantly different from the control value, and the final pH_i was not significantly different from the basal value (Table 2). However, ANP impaired both the stimulatory effect of AVP (at 10^{-12} and 10^{-9} mol/L, where during these situations, the final pH_i was not significantly different from the basal value; Table 2), as well as inhibited the effects of AVP (10^{-6} mol/L) on the net rate of pH_i recovery (but during this situation pH_i recovery was not complete; Table 2). These results indicate that ANP alone does not affect cellular pH recovery, but impairs both the stimulatory and inhibitory effects of AVP.

As some studies have shown the importance of cytosolic free calcium concentration for cellular H^+ secretion [13, 18], we studied the effect of addition of dimethyl-BAPTA-AM (50 μmol/L; an intracellular calcium chelator [13, 19]) to the medium on cellular pH recovery. Figure 4 also shows that with dimethyl-BAPTA-AM alone, the pH_i recovery rate was not significantly different from the control value (and the final pH_i was not significantly different from the basal value; Table 2). Dimethyl-BAPTA-AM impairs both stimulatory effects of AVP (10^{-12} and 10^{-9} mol/L) on the rate of pH_i recovery, and during both situations the final pH_i was not significantly different from the basal value (Table 2). Dimethyl-BAPTA-AM also impairs the inhibitory effect of AVP (10^{-6} mol/L) on the net rate of pH_i recovery despite that during this situation the pH_i recovery was not complete (Table 2). Taken together, these results suggest a role of cytosolic free calcium in regulating the net rate of pH_i recovery, mediated by Na^+/H^+ exchange and stimulated/impaired by AVP.

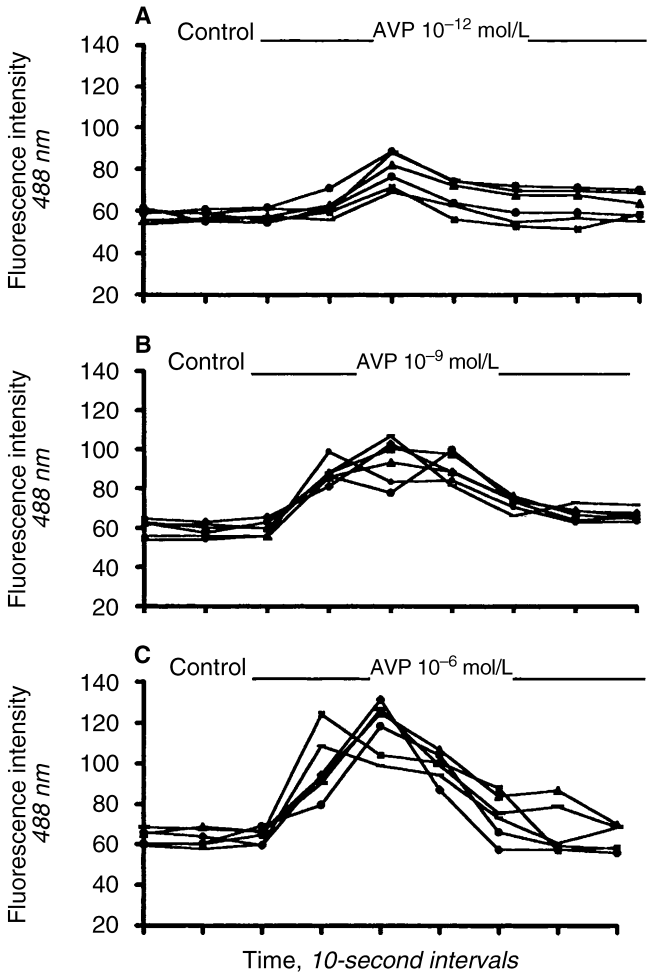

Fig. 5. Cell calcium fluorescent signal tracings during three representative experiments. The images were continuously acquired before and after addition of AVP (10^{-12}, 10^{-9}, or 10^{-6} mol/L), at time intervals of 10 seconds. The addition of AVP to the bath causes a significant and dose-dependent increase of the fluorescent signal.

$[Ca^{2+}]_i$

Figure 5 shows that the addition of AVP (10^{-12}, 10^{-9}, or 10^{-6} mol/L) to the bath of MDCK cells seeded on glass coverslips caused a significant increase of cell calcium fluorescent signal, in a dose-dependent manner. For each experiment, the maximum fluorescent signal for 10 cells was averaged and then used to calculate $[Ca^{2+}]_i$.

Figure 6 summarizes the main values of $[Ca^{2+}]_i$ found in all of the studied experimental groups. MDCK cells exhibited a mean baseline $[Ca^{2+}]_i$ of 100 ± 0.38 nmol/L ($N = 386$). The subsequent addition of AVP (10^{-12}, 10^{-9}, and 10^{-6} mol/L) increased $[Ca^{2+}]_i$ progressively from control values to 338 ± 1.4 nmol/L ($N = 25$), in a dose-dependent manner. The addition of ANP (10^{-6} mol/L) to the bathing solution leads to a rapid and significant decrease in $[Ca^{2+}]_i$ from control values to 40.8 ± 0.31 nmol/L ($N = 149$). In the presence of ANP, the subsequent addition of AVP (10^{-12}, 10^{-9}, and 10^{-6} mol/L) caused a recovery of $[Ca^{2+}]_i$ that reached 89.8 ± 1.8 nmol/L ($N = 26$), thus without exceeding normal baseline values even at AVP (10^{-6} mol/L). Figure 6 also shows that the addition of dimethyl-BAPTA-AM to the bathing solution leads to a significant decrease in $[Ca^{2+}]_i$ from control values to 50.5 ± 0.68 nmol/L ($N = 129$). In the presence of dimethyl-BAPTA-AM, the subsequent addition of AVP (10^{-12}, 10^{-9}, and 10^{-6} mol/L) caused a recovery of $[Ca^{2+}]_i$ to 112 ± 1.07 nmol/L ($N = 51$), 115 ± 0.76 nmol/L ($N = 43$) and 153 ± 1.87 nmol/L ($N = 37$), respectively.

DISCUSSION

The purpose of this study was to clarify the mechanism of interaction between AVP and ANP on the initial rate of pH_i recovery following acute intracellular acidification in Madin-Darby canine kidney (MDCK) cells, a permanent cell line originated from the renal collecting duct.

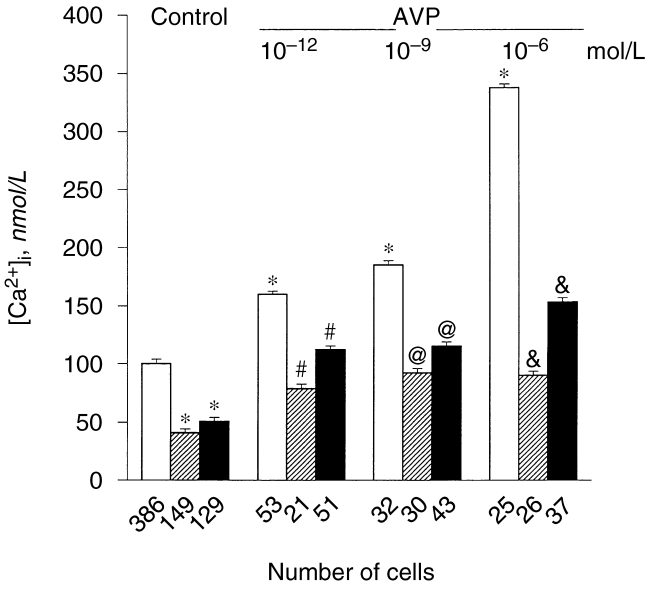

Fig. 6. Effects of AVP (10^{-12}, 10^{-9}, or 10^{-6} mol/L; □) and/or ANP (10^{-6} mol/L; ▨), and/or dimethyl-BAPTA/AM (50 μmol/L; ■) on free calcium concentration in the cytosol ($[Ca^{2+}]_i$) of MDCK cells. *$P <$ 0.05 vs. control (C); #$P < 0.05$ vs. AVP (10^{-12} mol/L); @$P < 0.05$ vs. AVP (10^{-9} mol/L); &$P < 0.05$ vs. AVP (10^{-6} mol/L).

In the present study, the MDCK cells were from passage 60 to 66, thus from cell strain I according to Richardson, Scalera and Simmons [20]. Our data demonstrate that MDCK cells in pH 7.4 HCO_3^--free solution maintain a mean baseline pH_i of 7.15 ± 0.005 ($N = 199$), a value compatible with the MDCK cell subtype C11 [21]. Our data are in accordance with the studies of Wiegmann et al [18], who used both fluorometry and video microscopy to show that MDCK cells had a mean pH_i of 7.12 ± 0.01 ($N = 50$). Our present results also agree with the value of 7.17 ± 0.01 ($N = 23$) found by Fernández and Malnic [22] in MDCK cells strain I, and with the value of 7.17 ± 0.04 ($N = 173$) found in our previous studies performed in MDCK cells [13].

Our data show that in the absence of external Na^+ the net rate of pH_i recovery was reduced to 34% of the control value (Fig. 2). This effect is partly reversed with the return of Na^+ to the bathing solution, indicating that the pH_i recovery is mostly dependent on Na^+/H^+ exchange (Table 2). This result is in accordance with Fernández and Malnic [22], who found three different mechanisms of pH_i recovery in MDCK cells: the Na^+/H^+ exchanger (the most important), the H^+-K^+ATPase, and the vacuolar H^+ATPase.

Our results indicate, to our knowledge for the first time in MDCK cells, that low concentrations (10^{-12} or 10^{-9} mol/L) of AVP stimulate and a high concentration (10^{-6} mol/L) of AVP inhibits the velocity of pH_i recovery (Fig. 2). In the absence of external Na^+, both stimulatory effects of AVP are significantly inhibited, and with the return of Na^+ to the bathing solution, they are subsequently partly recovered, indicating that they are mostly dependent on Na^+/H^+ exchange (Table 2).

In our previous studies using permeant filter supports, the Na^+/H^+ exchanger accounting for the Na^+-dependent pH_i recovery in MDCK cells was shown to be located on the basolateral membrane [13]. Our present data indicate that V1 or V2 receptors antagonists at the apical membrane surface do not affect the stimulatory and inhibitory effects of AVP on the velocity of pH_i recovery. However, V1 or V1 plus V2 receptor antagonists on the basolateral membrane surface return both the stimulatory and inhibitory effects of AVP to control levels (Fig. 3). In addition, the V2 receptor antagonist at the basolateral membrane surface converts the inhibitory effect of AVP to a stimulatory effect. Based on these data, it can be concluded that both the stimulatory and inhibitory effects of AVP on the basolateral Na^+/H^+ exchanger that account for the Na^+-dependent pH_i recovery are via activation of V1 receptors located on the basolateral membrane surface, and that basolateral V2 receptors have a dose-dependent inhibitor effect. In vivo experiments from our laboratory showed a luminal effect of AVP via V1 receptors, as opposed to the present results. This finding may be due to the use of rat tubules in the previous experiments rather than dog cells, and to differences between in vivo and cultured cells [8]. On the other hand, capillary perfusion experiments in the rat have shown the presence of both V1 and V2 receptors on the basolateral membrane of distal tubule cells (abstract; Musa-Aziz et al, *J Am Soc Nephrol* 11:7A, 2000).

In the present experiments on MDCK cells, ANP counteracted both the stimulating and the inhibiting effects of AVP (Fig. 4). These data are compatible with the identification of ANP receptors in MDCK cells [23]. Although only few ANP receptors have been found in the cortical distal tubule, such receptors are widely distributed in renal tissue, their mRNA having been detected in cortical and especially in medullary collecting duct cells [24]. Thus, it is possible that MDCK cells present properties more akin to the medullary collecting duct with respect to these receptors. On the other hand, these results are very similar to those we obtained with Ang II in MDCK cells: ANP (10^{-6} mol/L) counteracted both the stimulating and the inhibiting effects of Ang II on the net rate of Na^+-dependent pH_i recovery [13]. In addition, an interaction between ANP and AVP has been observed in other tissues: ANP inhibits cAMP synthesis stimulated by AVP in rat renal papillary collecting tubule cells in culture [12], and AVP stimulated Na^+/H^+ exchange in vascular smooth muscle cells in culture [11].

To obtain information on the mechanism of the interaction of these hormones on pH_i regulation, we studied their effects on the regulation of $[Ca^{2+}]_i$. Our results indicate that MDCK cells exhibited a mean baseline

$[Ca^{2+}]_i$ of 100 ± 0.38 nmol/L ($N = 386$). These data agree with the value of 120 ± 29 nmol/L ($N = 6$) found by Borle and Bender [25] or of 125 ± 7 nmol/L ($N = 50$) found by Weigmann et al in MDCK cells [18]. This value also is not significantly different from the basal value of $[Ca^{2+}]_i$ monitored with the fluorescent probe Fura-2 in these cells while in suspension, as described in our previous study (99.0 ± 10 nmol/L, $N = 20$) [13].

Our data show that $[Ca^{2+}]_i$ increases progressively as AVP concentrations increase from 10^{-12} to 10^{-6} mol/L (Figs. 5 and 6). These results are in accordance with data from the literature. It has been proposed that V1 receptors mediate AVP action mostly via a G_{q11} protein-phospholipase C-IP_3-protein kinase C-Ca^{2+} pathway [6, 26–28]. Besides, it is known that protein kinase C, via phosphorylation, may stimulate the Na^+/H^+ exchanger [29]. This behavior is compatible with our data showing that low concentrations of AVP stimulate the rate of Na^+-dependent pH_i recovery via V1 receptors (Fig. 3).

At high concentrations, AVP is known to interact with V1 receptors, causing the liberation of arachidonic acid, which is part of a path that elevates cell calcium by activating voltage-sensitive calcium channels of the plasma membrane [27]. At high cytosolic concentrations, calcium may inhibit Na^+/H^+ exchange by activating Na^+/Ca^{2+} exchange at the cell membrane, thereby increasing cell sodium, which decreases the gradient responsible for H^+ extrusion by the exchanger [29, 30]. This behavior is compatible with our data showing that (*1*) the effect on the rate of Na^+-dependent pH_i recovery with AVP (10^{-12} mol/L) is higher than with AVP (10^{-9} mol/L), but in the presence of V1 antagonist is similar for both doses, and (*2*) AVP (10^{-6} mol/L) inhibits the rate of Na^+-dependent pH_i recovery via V1 receptors (Fig. 3).

On the other hand, it is well known that V2 receptors are present mostly at the basolateral membrane, where they mediate the hydro-osmotic effect of AVP at picomolar concentrations. This mechanism is known to involve a dose-dependent adenylate cyclase, cAMP, protein kinase A pathway that, at high AVP concentrations, is expected to inhibit the Na^+/H^+ exchanger [31]. This behavior also is compatible with our present data, since in the presence of a V2 receptor antagonist on the basolateral membrane surface, the inhibitory effect of 10^{-6} mol/L AVP on the rate of pH_i recovery is converted to a stimulatory effect that is significantly higher than the control or 10^{-6} mol/L AVP plus V1 receptor antagonist values (Fig. 3).

Our results show that when 10^{-6} mol/L ANP is added to the bathing solution, $[Ca^{2+}]_i$ decreases to approximately 41% of the control value. In the presence of ANP, the subsequent addition of AVP (from 10^{-12} to 10^{-6} mol/L) caused a recovery of $[Ca^{2+}]_i$ without exceeding normal baseline values even at AVP 10^{-6} mol/L (Fig. 6). Our results also show that ANP alone does not affect the rate of Na^+-dependent pH_i recovery, but ANP impairs both the stimulatory and inhibitory effects of AVP in this process (Fig. 4). These data are compatible with our previous results in MDCK cells showing that—in contrast to EGTA—ANP alone does not affect the rate of Na^+-dependent pH_i recovery, since it causes only a moderate decrease of cytosolic free calcium as compared to the minimal $[Ca^{2+}]_i$ values found in presence of EGTA (to 35 and 15% of the control value, respectively) [13]. On the other hand, ANP impairs both stimulatory and inhibitory effects of AVP on the rate of Na^+-dependent pH_i recovery because it impairs the increase of $[Ca^{2+}]_i$ in response to AVP, thus modulating the cellular action of AVP. It is possible that this is the mechanism by which ANP inhibits the AVP-stimulated Na^+/H^+ exchange in vascular smooth muscle cells in culture [11], and impairs both the stimulatory and inhibitory effects of Ang II on the rate of Na^+-dependent pH_i recovery in MDCK cells [13].

This behavior is also in agreement with the results concerning the effect of dimethyl-BAPTA-AM on the rate of pH_i recovery. Similar to ANP, dimetyl-BAPTA/AM alone does not affect the rate of pH_i recovery since, like ANP, it causes only a moderate decrease (to about 51%) of cytosolic free calcium (Fig. 6). On the other hand, like ANP, dimethyl-BAPTA-AM impairs both stimulatory and inhibitory effects of AVP on the rate of pH_i recovery since, like ANP, it impairs the increase of $[Ca^{2+}]_i$ in response to AVP. In addition, with dimethyl-BAPTA-AM plus AVP (10^{-6} mol/L), the $[Ca^{2+}]_i$ values are not significantly different from AVP (10^{-12} mol/L) values [153 ± 1.87 ($N = 37$) and 160 ± 1.45 ($N = 53$) nmol/L, respectively], but the pH_i recovery values measured in the presence of dimethyl-BAPTA-AM plus AVP 10^{-6} mol/L are significantly smaller than the values found in presence of AVP 10^{-12} mol/L (Fig. 4), because in the presence of high doses of AVP an inhibitory effect occurs on the Na^+/H^+ exchange mediated by cAMP-protein kinase A, via V2 receptors.

In conclusion, the results obtained in our studies suggest that $[Ca^{2+}]_i$ has a role in regulating the process of pH_i recovery after the acid load induced by NH_4Cl, which is mediated by the basolateral Na^+/H^+ exchanger and stimulated/impaired by AVP. The data are compatible with stimulation of the Na^+/H^+ exchanger by increases of $[Ca^{2+}]_i$ in the lower range (at 10^{-12} or 10^{-9} mol/L AVP; mediated by basolateral V1 receptors via a G_{q11} protein phospholipase C IP_3 protein kinase C–Ca^{2+} pathway) and inhibition at high $[Ca^{2+}]_i$ levels (at 10^{-6} mol/L AVP; via activation of basolateral V1 receptors causing the liberation of arachidonic acid). They are also compatible with inhibition of the Na^+/H^+ exchanger at high cell cAMP levels (at 10^{-6} mol/L AVP, mediated by basolateral V2 receptors). While ANP and dimethyl-BAPTA/AM cause a moderate decrease of $[Ca^{2+}]_i$, this does not affect the pH_i recovery, but, by impairing the path causing the

increase in $[Ca^{2+}]_i$, they block both the stimulatory and inhibitory effects of AVP during this process. The question of whether $[Ca^{2+}]_i$ modification represents an important direct mechanism for exchanger activation or is a side effect of other signaling pathways must await additional studies.

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