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ADH Action on Whole-Cell Currents by Cytosolic Ca^{2+}-dependent Pathways in Aldosterone-treated A6 Cells

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Abstract. We studied the characteristics of the basal and antidiuretic hormone (arginine vasotocin, AVT)-activated whole cell currents of an aldosterone-treated distal nephron cell line (A6) at two different cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$, 2 and 30 nM). A6 cells were cultured on a permeable support filter for 10 ~ 14 days in media with supplemental aldosterone (1 μM). At 30 nM $[Ca^{2+}]_c$, basal conductances mainly consisted of Cl^- conductances, which were sensitive to 5-nitro-2-(3-phenylpropylamino)-benzoate. Reduction of $[Ca^{2+}]_c$ to 2 nM abolished the basal Cl^- conductance. AVT evoked Cl^- conductances at 2 as well as 30 nM $[Ca^{2+}]_c$. In addition to Cl^- conductances, AVT induced benzamil-insensitive nonselective cation (NSC) conductances. This action on NSC conductances was observed at 30 nM $[Ca^{2+}]_c$ but not at 2 nM $[Ca^{2+}]_c$. Thus, cytosolic Ca^{2+} regulates NSC and Cl^- conductances in a distal nephron cell line (A6) in response to AVT. Keeping $[Ca^{2+}]_c$ at an adequate level seems likely to be an important requirement for AVT regulation of ion conductances in aldosterone-treated A6 cells.

Key words: Patch clamp — Ion channel — Amiloride — Benzamil — NPPB

Introduction

It is widely accepted that A6 cells, an epithelial cell line isolated from *Xenopus* kidney (Rafferty, 1969), have the characteristics of a distal nephron epithelium (Perkins & Handler, 1981; Benos et al., 1986; Palmer, 1992). A6 cells cultured on a permeable support filter form tight junctions and can be used as a model of the distal nephron for studies on ion transport and its regulation by hormones such as antidiuretic hormone (ADH) and aldosterone using short-circuit current measurement (Bindels, Schafer & Reif, 1988; Wills & Millinoff, 1990; Wills, Millinoff & Crowe, 1991; Kemendy, Kleyman & Eaton, 1992; Schafer & Hawk, 1992; Chalfant, Coupaye-Gerard & Kleyman, 1993; Schafer, 1994; Doi & Marunaka, 1995) and patch clamp techniques (Marunaka & Eaton, 1990*b;* Marunaka & Eaton, 1990*a;* Marunaka et al., 1991; Marunaka, Hagiwara & Tohda, 1992; Schafer & Hawk, 1992; Marunaka & Tohda, 1993; Schafer, 1994; Marunaka & Tohda, 1994).

ADH is known to increase the reabsorption of Na^+ in the distal nephron by increasing apical membrane permeability to Na^+ (Perkins & Handler, 1981; Bindels et al., 1988; Marunaka & Eaton, 1991; Smith & Benos, 1991; Palmer, 1992; Schafer & Hawk, 1992; Kemendy et al., 1992; Chalfant et al., 1993; Schafer, 1994). Our previous studies (Marunaka et al., 1994; Nakahari & Marunaka, 1995) have demonstrated that AVT activates nonselective cation (NSC) channels in the apical membrane of aldosterone-untreated A6 cells at single channel and whole cell current levels. Further, we have reported that the ADH action on ion transport in A6 cells is modified by aldosterone treatment (Doi & Marunaka, 1995). In the present study, we studied the characteristics of the basal whole cell current and ADH action on whole cell current in A6 cells treated with aldosterone. Many studies on the role of increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in ion transport have been performed, but the role of basal $[Ca^{2+}]_c$ has not been studied. The purpose of the present study was to clarify the role of the basal level of $[Ca^{2+}]_c$ in ADH action on whole cell currents in A6 cells treated with aldosterone. Our preliminary ob-

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Table 1. Composition of bath solutions (mM)

Solution	A	B	C	D
Na	122.5	122.5	122.5	
K	3.5	3.5		
Cl	125	125	4	125
Mg	1	1	1	1
Ca	1		1	1
EGTA		1		
NMDG				121
Gluconate			122.5	
HEPES	10	10	10	10
Glucose	5	5	5	5

The pH was adjusted at 7.4 by NaOH for A, B, and C, and HCl for D.

Table 2. Composition of pipette solutions (mM)

	(K^+ solution)		(Na^+ solution)		(NMDG solution)	
Solution	E	F	G	H	I	J
Na			105	105		
K	105	105				
Cl	105		105		105	
Mg	2	2	2	2	2	2
Ca						
EGTA	10	10	10	10	10	10
NMDG					105	105
Gluconate		105		105		105
HEPES	10	10	10	10	10	10
Glucose	5	5	5	5	5	5
ATP	2	2	2	2	2	2

The pH was adjusted at 7.4 by KOH for K^+ solution, NaOH for Na^+ solution, HCl or gluconic acid for NMDG solution.

servations suggest that $[Ca^{2+}]_c$ in A6 cells is about 30 nM. Therefore, we measured the whole cell currents when $[Ca^{2+}]_c$ was 30 nM and studied the effect of reduction of $[Ca^{2+}]_c$ on the whole cell currents.

Materials and Methods

A6 cells were purchased from American Type Culture Collection (Rockville, MD) in the 68th platings. A6 cells were maintained in plastic culture flasks at 27°C in an incubator with 4% CO_2 in air. All the experiments were performed on the 75th to 83rd platings. The culture medium was NCTC-109 medium (GIBCO, Grand Island, NY) modified for amphibian cells (100 mM NaCl and 20 mM $NaHCO_3$, pH 7.4), in which 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY), 10 mg/ml streptomycin and 10,000 U/ml penicillin (Irvine Scientific, Santa Ana, CA) were added. For the patch clamp experiments, A6 cells were subcultured for 10 ~ 14 days on a permeable suport filter (Nunc Tissue Culture Inserts, Nunc, Naperville, IL) to form a polarized monolayer. Aldosterone (the final concentration = 1 μM) dissolved with ethanol was added into the medium for 3 days prior to patch experiments. Ethanol concentration never exceeded 0.1%. A6 cell is an amphibian cell line and the effective aldosterone level in amphibian is reported to be higher than in mammalian kidney (Fanestil & Park, 1981). Our previous studies have demonstrated that aldosterone at the level in FBS does not have any effects on short-circuit currents or whole cell currents in A6 cells (Nakahari & Marunaka, 1995; Doi & Marunaka, 1995).

The compositions of the bathing and pipette solutions (pH 7.4) are summarized in Tables 1 and 2. Solution A was used as a control solution. For Cl^--free or Na^+/K^+-free experiments, solution C or D was used, respectively. To obtain an isolated single cell, we incubated cells for 40 min in solution B which contained EGTA (1 mM) at 27°C. Then, the cells were incubated in solution A for 15 min at 27°C. Subsequent to this incubation in solution A, the isolated single cell was used for the patch-clamp experiments within 30 min after 15 min incubation in solution A.

To vary the pipette Ca^{2+} concentration ($[Ca^{2+}]_c$, 2 nM and 30 nM), the appropriate amounts of $CaCl_2$ were added into the pipette solution containing 10 mM EGTA. The free Ca concentration was adjusted using the known $CaCl_2$ and EGTA (10 mM as pure EGTA) concentrations, calculated with pK_d values of 10.86 for $EGTA^{4-}$ and 5.25 for $HEGTA^{3-}$. To fix the pipette Cl^- concentration ($[Cl^-]_c$) at 50 mM, we mixed appropriate amounts of solutions E and F for K^+ solution, solutions G and H for Na^+ solution, and solutions I and J for *N*-methyl-D-glucamine (NMDG) solution.

AVT and ATP were purchased from Sigma Chemical (St. Louis, MO). Benzamil was a gift from Merck Sharp & Dohme Research Labortory (West Point, PA). 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) was a generous gift from Prof. R. Greger (Germany). AVT of 70 nM (35 mU/ml), benzamil of 1 μM and NPPB of 20 μM were used in the present study. These compounds were dissolved with dimethyl sulfoxide (DMSO), whose final concentration was less than 0.1%.

We used a standard whole cell patch-clamp technique to measure whole cell currents (Marty & Neher, 1993). Patch pipettes were prepared from LG-16 glass (Dagan, Minneapolis, MN) and were fire polished. When they were filled with a K^+pipette solution and immersed in solution A, their resistances were about 5 MΩ. During the whole-cell current measurements, negative pressure (5–10 cm H_2O) was applied in the pipette, because the level of the pipette solution was higher than that of bathing solution and a cessation of the continuous suction provided positive pressure due to the hydrostatic pressure of the pipette solution (+5 cm H_2O) for increasing cell volume (Doroshenko & Neher, 1992). The electrical measurements were performed with an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA). Whole cell currents were directly stored into a computer via an analog-to-digital converter (TL-1 DMA interface, Axon Instruments). The cell membrane was held at a potential of −20 mV. The command potentials for the whole-cell voltage clamp ranged from −100 to 100 mV in 20 mV intervals, and the duration was 90 msec (Fig. 1). The whole cell currents were measured at 70 to 90 msec after the onset of voltage pulse. The experiments were performed at room temperature (22 ~ 23°C). The whole-cell currents are expressed as means ± SD of 4 ~ 6 experiments.

The paired and unpaired *t*-test was used for statistical analysis as appropriate and the *P* value <0.05 was considered significant.

Results

Whole-Cell Currents at 30 nM $[Ca^{2+}]_c$

Figure 1 shows actual current traces before (Fig. 1*A*) and 4 min after application of AVT ((Fig. 1*B*) when the pi-

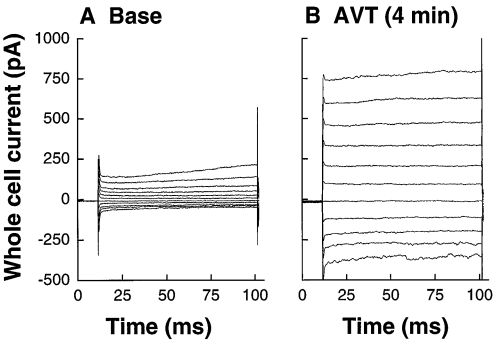

Fig. 1. Whole cell currents of A6 cells. The command potential for the whole cell voltage clamp ranged from −100 to 100 mV in 20 mV intervals, and the duration was 90 msec. The pipette solution was K^+ solution containing 30 nM Ca^{2+} and 50 mM Cl^-, and the bathing solution was solution A (NaCl solution) (*see* Tables 1 and 2). (*A*) Base (without AVT application). (*B*) 4 min after addition of AVT.

pette solution was the K^+ solution containing 50 mM Cl^- and 30 nM Ca^{2+} and the bathing solution was Solution A (NaCl). The whole-cell current increased in response to AVT. Figure 2*A* shows the time course of AVT action at command potentials of +80 and −80 mV. The whole-cell currents reached a peak about 4 min after addition of AVT, and then they decreased gradually (Fig. 2*A*). The current-voltage (*I–V*) relationships before and after application of AVT showed outward rectification (*see* Fig. 2*B*). The reversal potentials were respectively ~−20 mV before and ~−10 mV after application of AVT.

The basal current was not affected by replacement of Na^+ (closed circles in Fig. 3) with K^+ (open circles in Fig. 3) in the pipette, although the reversal potential shifted from 0 to −10 mV. Next, Na^+ and K^+ in the bathing and pipette solution were replaced with NMDG. Under this condition, the major permeable ion was only Cl^- and the reversal potential was very close to the equilibrium potential for Cl^- (E_{Cl}) (open triangles in Fig. 3). However, the values of the basal currents were not largely changed by replacement of Na^+ and K^+ with NMDG, although the inward currents slightly decreased (compare open triangle with closed circle in Fig. 3). These observations suggest that the Cl^- conductance contributes to the basal conductance and even though the cation conductances contribute to the basal whole cell conductances, they are very small. The AVT-evoked currents were also not significantly changed by replacement of Na^+ with K^+ in the pipette solutions (compared closed squares (Na^+) with open squares (K^+) in Fig. 3). On the other hand, the replacement of Na^+ and K^+ with NMDG significantly decreased the AVT-evoked currents (compared closed triangles (NMDG) with closed squares (Na^+) or open squares (K^+) in Fig. 3) and the reversal potential was very closed to E_{Cl} (closed triangles (NMDG) in Fig. 3). These observations suggest that the AVT-evoked currents were generated by NSC and Cl^- conductances.

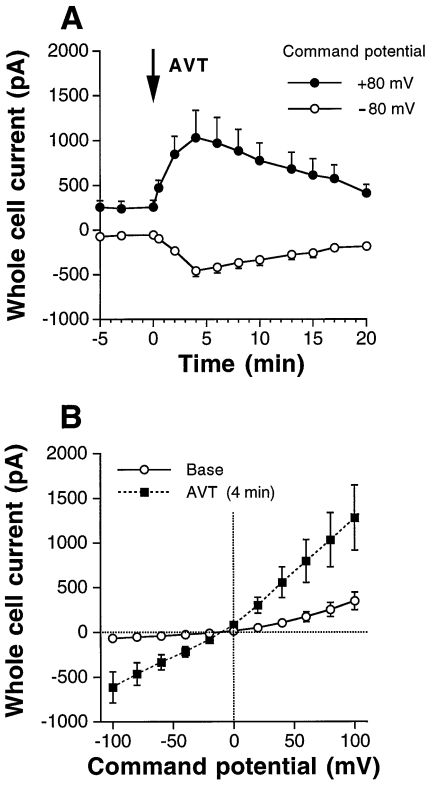

Fig. 2. The whole-cell currents activated by AVT in 30 nM $[Ca^{2+}]_c$. The pipette solution was K^+ solution containing 30 nM Ca^{2+} and 50 mM Cl^-, and the bathing solution was solution A (NaCl solution). (*A*) The whole-cell currents at the command potentials of ±80 mV (●, 80 mV; ○, −80 mV) are plotted against time. AVT rapidly increased the whole-cell currents, which gradually decreased after reaching its peak. (*B*) Effects of AVT on *I-V* relationship (○, the basal current; ■, the current at 4 min after addition of AVT). AVT increased both inward and outward whole-cell currents.

Whole-Cell Currents in 2 nM $[Ca^{2+}]_c$

When $[Ca^{2+}]_c$ was 2 nM and the pipette solution was KCl (K^+ solution, $[Cl^-]_c$ = 50 mM) and the bathing solution was NaCl (Solution A), the basal whole-cell currents (closed circles in Fig. 4) were very small. Even though Na^+ and K^+ in the bathing and pipette solutions were replaced with NMDG (open circles in Fig. 4), the basal whole currents were not affected. The reversal potentials were closed to 0 mV under both conditions. The level of current was similar to the leak current (*see* detail in the Discussion). This suggests that the basal membrane conductance was negligibly small in 2 nM $[Ca^{2+}]_c$. AVT increased the whole-cell currents and the reversal potential shifted to E_{Cl} even in the presence of Na^+ and K^+ in the bathing and pipette solutions (closed squares in Fig. 4). This phenomenon was not significantly changed by replacement of Na^+ and K^+ with NMDG (open squares in Fig. 4). These observations suggest that AVT-evoked

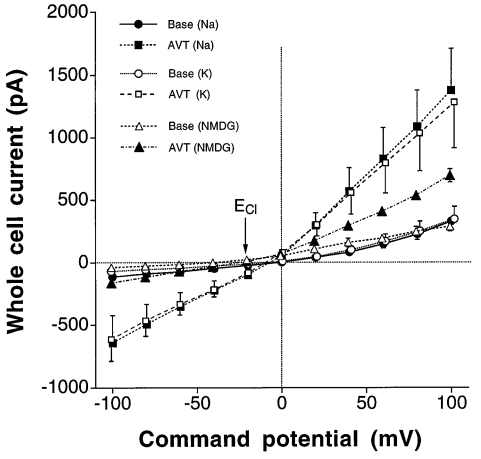

Fig. 3. Effects of AVT on whole-cell current in 30 nM $[Ca^{2+}]_c$. The pipette solution contained 30 nM Ca^{2+} and 50 mM Cl^-. The basal and AVT-evoked currents in Na^+ solutions (the bathing solution, Solution A (NaCl solution); the pipette solution, Na^+ solution) are respectively shown by closed circles (●, base) and closed squares (■, at 4 min after application of AVT). The basal and AVT-evoked currents in K^+ pipette solution (the bathing solution, Solution A (NaCl solution); the pipette solution, K^+ solution) are respectively shown by open circles (○, base) and open squares (□, at 4 min after application of AVT). The basal and AVT-evoked currents in NMDG solutions (the bathing solution, Solution D (NMDG-Cl solution); the pipette solution, NMDG-Cl solution) are respectively shown by open triangles (△, base) and closed triangles (▲, at 4 min after application of AVT).

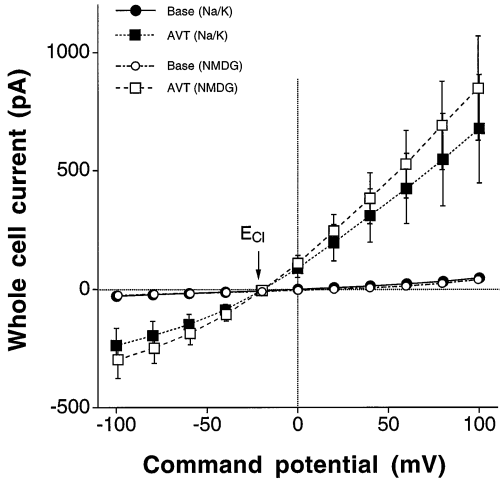

Fig. 4. Effects of AVT on whole-cell currents in 2 nM $[Ca^{2+}]_c$. The basal and AVT-evoked currents in the Na^+ bathing solution (Solution A, NaCl solution) with the K^+ pipette solution (K^+ solution) are respectively shown by closed circles (●, base) and closed squares (■, at 4 min after application of AVT). The basal- and AVT-evoked currents in NMDG solutions (the bathing solution, Solution D (NMDG-Cl solution); the pipette solution, NMDG-Cl solution) are respectively shown by open circles (○, base) and open squares (□, at 4 min after application of AVT).

currents are due to an increase in the Cl^- conductance. Thus, the cation conductances contribute to neither basal nor AVT-evoked currents at 2 nM $[Ca^{2+}]_c$, while AVT evoked only the Cl^- conductance.

Effects of $[Ca^{2+}]_c$ on the basal and AVT-evoked Whole-Cell Currents

A rise in $[Ca^{2+}]_c$ significantly increased both the outward and inward currents in the normal solution (the bathing solution, Solution A (NaCl); the pipette solution, K^+ solution; Fig. 5*A*), and this phenomenon could also be observed in NMDG solution (Fig. 5*B*) to a similar extent to that in the normal solution (Fig. 5*A*). This suggests that the basal Cl^- conductance depends on $[Ca^{2+}]_c$. Further, this Ca^{2+}-dependent Cl^- current shows marked outward rectification.

The AVT-evoked currents also depended on $[Ca^{2+}]_c$. A rise in $[Ca^{2+}]_c$ increased the currents in the normal solution (the bathing solution, Solution A (NaCl); the pipette solution, K^+ solution; Fig. 6*A*) but not in NMDG solution (Fig. 6*B*). This indicates that the $[Ca^{2+}]_c$-dependent AVT-evoked currents carried cation but are not Cl^- currents. Namely, AVT evoked the NSC currents in a $[Ca^{2+}]_c$-dependent manner, while the AVT-evoked Cl^- currents were not dependent on $[Ca^{2+}]_c$ (2 or 30 nM).

Tables 3 and 4 summarize the basal and AVT-evoked currents. Namely, basal Cl^- conductances require normal $[Ca^{2+}]_c$, however AVT-activated Cl^- conductances do not require normal $[Ca^{2+}]_c$ (Table 3). On the other hand, NSC conductances are only observed in AVT-activated cells and require normal $[Ca^{2+}]_c$ (Table 4).

Effects of Ion Channel Blockers on the Currents

We further studied the effects of channel blockers (Ba^{2+}, benzamil and NPPB) on the basal and AVT-evoked currents. Figure 7 shows the effects of benzamil, Ba^{2+} and NPPB on the basal currents in 30 nM $[Ca^{2+}]_c$. Although under this condition the effects of 2 mM Ba^{2+} on the whole cell currents were unclear (Fig. 7*A*), the reversal potential shifted toward positive potentials by 10 mV after addition of Ba^{2+}, suggesting that Ba^{2+} seems likely to block the basal K^+ currents. Benzamil (1 μM) decreased inward currents (Fig. 7*B*). NPPB (20 μM) suppressed the whole cell currents (Fig. 7*C*). Figure 7*D* shows the effects of these channel blockers on the basal currents at ±80 mV. These observations indicate that the basal conductance of A6 cells is at least composed of benzamil-sensitive and NPPB-sensitive conductances.

We also studied the effect of benzamil on the AVT-evoked currents in 30 nM $[Ca^{2+}]_c$, since these currents contained NSC currents. Figure 8*A* shows the control experiments in the absence of extracellular Cl^- (only 4

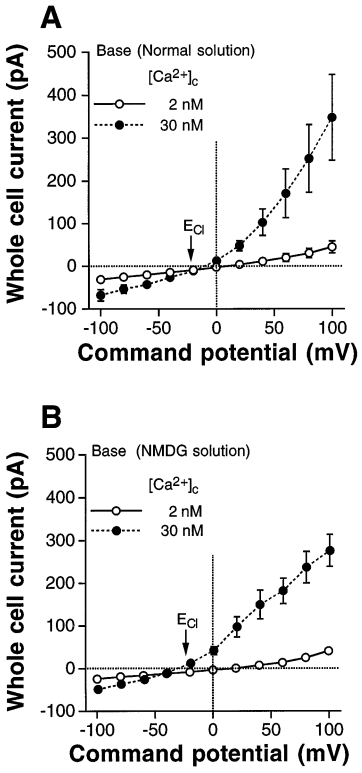

Fig. 5. Effects of $[Ca^{2+}]_c$ on the basal whole-cell currents (○, 2 nM $[Ca^{2+}]_c$; ●, 30 nM $[Ca^{2+}]_c$). The $[Cl^-]_c$ was fixed at 50 mM. (*A*) K^+ pipette and Na^+ bathing solutions. (*B*) NMDG pipette and bathing solutions.

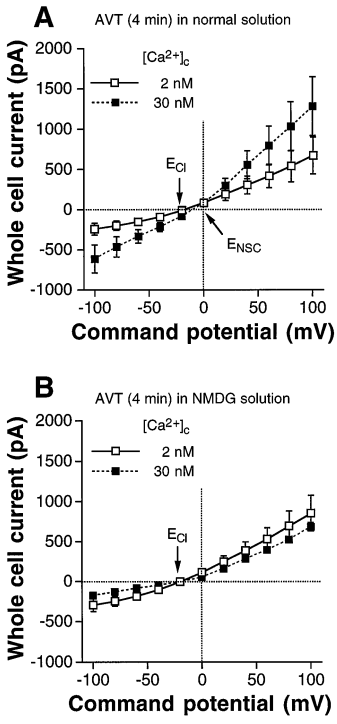

Fig. 6. Effects of $[Ca^{2+}]_c$ on the AVT-activated whole-cell currents (□, 2 nM $[Ca^{2+}]_c$; ■, 30 nM $[Ca^{2+}]_c$). The $[Cl^-]_c$ was fixed at 50 mM. (*A*) K^+ pipette and Na^+ bathing solutions. (*B*) NMDG pipette and bathing solutions.

mM) without benzamil. Application of benzamil to the bathing solution decreased the basal currents by a small amount (Fig. 8*B*), however AVT evoked the current (Fig. 8*B*) to a similar extent as control (Fig. 8*A*). This suggests that the AVT-evoked NSC conductance is insensitive to benzamil. Finally, we examined whether the AVT-evoked Cl^- current is blocked by NPPB. As shown in Fig. 9, NPPB blocked the AVT-evoked Cl^- currents.

Discussion

The AVT-evoked whole cell currents consisted of two components in 30 nM $[Ca^{2+}]_c$; i.e., NSC and Cl^- currents. The AVT-evoked NSC current was abolished by reduction of $[Ca^{2+}]_c$ to 2 nM, while the AVT-evoked Cl^- current was not affected by the reduction of $[Ca^{2+}]_c$. On the other hand, basal currents were due to Cl^- conductances, and were abolished by reduction of $[Ca^{2+}]_c$.

A6 cells were treated with 1 mM EGTA-containing solution (Solution B + 1 mM EGTA) for 40 min prior to patch experiments, subsequently incubated in a control solution (Solution A) for 15 min. So, although the cells had the polarity of apical and basolateral membranes before exposure to EGTA, it is unclear how long the cells maintained the polarity during the experimental period. The cells might lose the polarity. However, the purpose of the present study was to characterize the whole-cell

Table 3. AVT action on Cl^- conductances at 2 and 30 nM $[Ca^{2+}]_c$

	Base	AVT
$[Ca^{2+}]_c$		
2 nM	−	++
30 nM	+	++

−, no conductances were observed; +, some conductances were observed; ++, larger conductances were observed compared with +.

Table 4. AVT action on nonselective cation conductances at 2 and 30 nM) $[Ca^{2+}]_c$

	Base	AVT
$[Ca^{2+}]_c$		
2 nM	−	−
30 nM	−	+

−, no conductances were observed;
+, some conductances were observed.

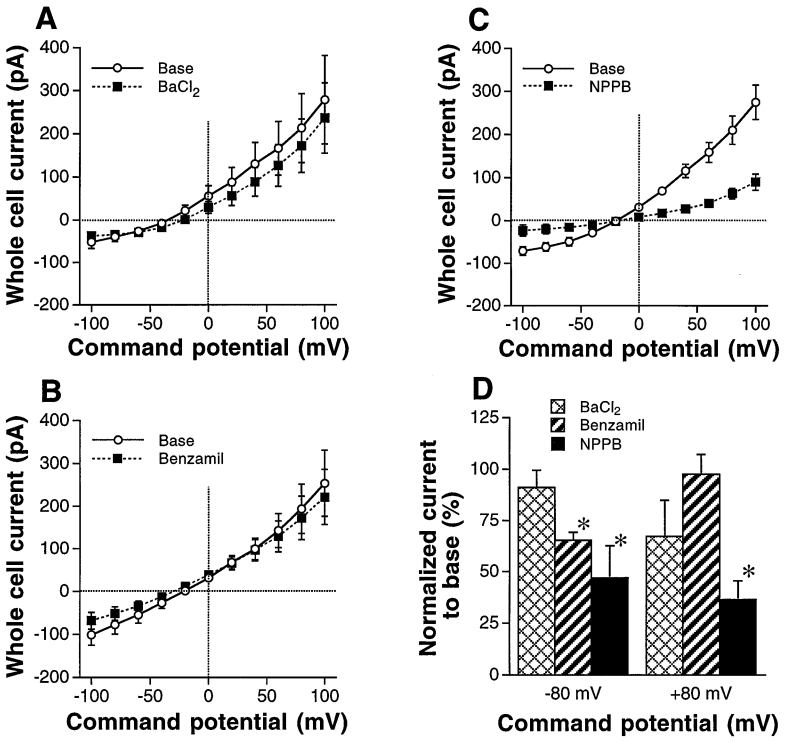

Fig. 7. Effects of ion channel blockers on the basal whole-cell currents. The pipette solution was K^+ solution containing 30 nM Ca^{2+} and 50 mM Cl^-, and the bathing solution was solution A (NaCl solution) with (■) and without (○) of blocker. (*A*) The effects of $BaCl_2$ (2 mM). The reversal potential was shifted to positive side by application of $BaCl_2$. (*B*) The effects of benzamil (an analogue of amiloride, 1 μM). Benzamil suppressed only inward currents. (*C*) The effects of NPPB (20 μM). NPPB markedly suppressed whole cell currents. (*D*) The effects of blockers are summarized. The percentages of control currents in each experiments are shown as % of control. The holding potentials were ±80 mV. *, $P < 0.05$.

conductance. So, the information about the whole-cell conductances shown in the present study is still useful for study in ion transport and its regulation in A6 cells, although keeping the polarity is very important to determine the localization of ion conductances.

The AVT-evoked large Cl^- conductance described in the present study was not observed in A6 cells without aldosterone treatment, although we observed small increases in Cl^- conductances evoked by AVT in aldosterone-untreated A6 cells (Nakahari & Marunaka, 1995). In the present study, we could detect the AVT-evoked NSC conductance in 30 nM $[Ca^{2+}]_c$ but not in 2 nM $[Ca^{2+}]_c$. Our previous study (Nakahari & Marunaka, 1995) indicated that AVT evoked NSC conductances in aldosterone-untreated A6 cells with 2 nM $[Ca^{2+}]_c$. These observations suggest that aldosterone treatment diminishes the AVT-evoked NSC conductance when $[Ca^{2+}]_c$ is low. Our previous study (Marunaka et al., 1994) reported that elevation of $[Ca^{2+}]_c$ decreased the activity of NSC channels in aldosterone-untreated A6 cells. If the AVT-evoked NSC currents shown in the present study are due to the same channels as those in aldosterone-untreated cells (Marunaka et al., 1994), the observation reported here seems inconsistent with our previous observation. However, in the previous report we only studied the dependency of the NSC channels on $[Ca^{2+}]_c$ in excised inside-out configuration with 30 nM ~ 1 μM $[Ca^{2+}]_c$ (not <30 nM). So, to clarify the relationship between single-channel and whole-cell currents, we will need further studies to examine the effects of $[Ca^{2+}]_c$ on the NSC channels with cell-attached configurations with wider ranges of $[Ca^{2+}]_c$.

Three types of Cl^- channels have been reported in the apical membrane of A6 cells (Nelson, Tang & Palmer, 1984; Marunaka & Eaton, 1990*b*). Two of them with single-channel conductances of 3 and 8 pS have been shown to respond to ADH (Marunaka, 1993; Marunaka & Tohda, 1993, 1994). The 8 pS Cl^- channel is Ca^{2+}-insensitive, and its *I-V* relationship is linear. The open probability of this channel is independent of voltage. However, the number of the channels was increased by AVT (Marunaka & Tohda, 1993, 1994). Therefore, the *I-V* relationship of the AVT-evoked whole cell currents due to the 8 pS Cl^- channel should be linear. Another type of Cl^- channel (3 pS) is activated by cytosolic Ca^{2+}, and its *I-V* relationship outwardly rectifies. ADH alters the Ca^{2+} sensitivity of this 3 pS Cl^- channel, and increases the open probability in voltage-dependent fashion (Marunaka, 1993; Marunaka & Tohda, 1994). Therefore, activation of 3 pS Cl^- channels by AVT may explain the observation that the AVT-evoked current has an outwardly rectifying *I-V* relationship. The Cl^- channel with the largest single channel conductance (360 pS) (Nelson et al., 1984) was not observed after A6 cells became confluent (our preliminary observation), suggesting that this channel would not contribute to the Cl^- conductance in confluent A6 cells. In addition to the apical Cl^- channels, however, we should consider the basolateral Cl^- conductances. A6 cells have been reported to have a large basolateral Cl^- conductance in the

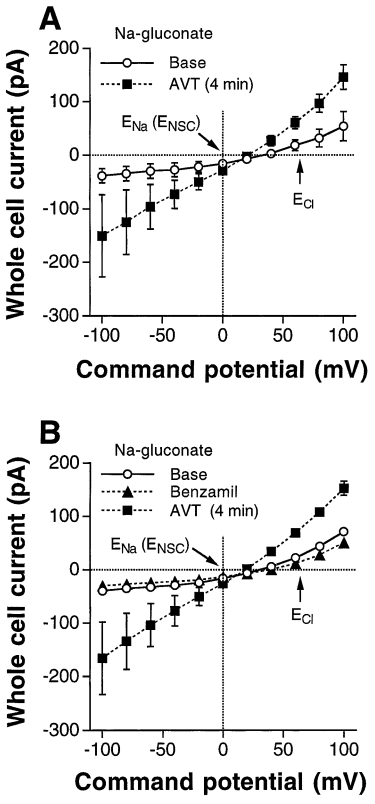

Fig. 8. Effects of benzamil on the AVT-activated whole-cell current in 30 nM $[Ca^{2+}]_c$. The pipette solution was Na^+ solution, whose $[Ca^{2+}]$ and $[Cl^-]$ were respectively fixed at 30 nM and 50 mM, and the bathing solution was solution C (Na-gluconate solution). Under this condition the outward currents were generated only by Na^+ and the inward currents were by both Na^+ and Cl^-. (*A*) Effects of AVT on the whole-cell currents. AVT increased both inward and outward currents (○, the basal current; ■, the current at 4 min after application of AVT). (*B*) The whole-cell current activated by AVT in benzamil-treated A6 cells (○, base; ▲, benzamil (1 μM), ■: AVT in the presence of benzamil). In the case of K^+ pipette solution we observed similar results. These suggest that AVT activated benzamil (amiloride)-insensitive NSC channels.

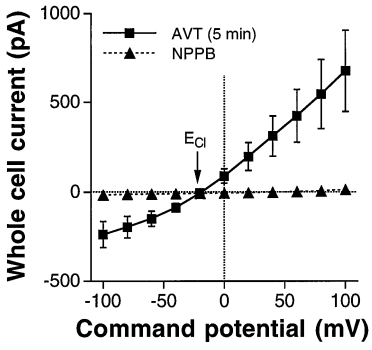

Fig. 9. Effects of NPPB on the AVT-activated whole-cell current. The pipette solution was K^+ pipette solution. The $[Ca^{2+}]_c$ and $[Cl^-]_c$ were respectively fixed at 30 nM and 50 mM. AVT increased whole cell currents (■, at 5 min after addition of AVT). NPPB almost completely suppressed the AVT-activated whole cell current (▲).

basolateral membrane (Granitzer, Nagel & Crabbe, 1992). Nilius et al. (1995) have reported that A6 cells have a 36 pS Cl^- channel. We could not detect the 36 pS Cl^- channel in the apical membrane, so the 36 pS Cl^- channel may be localized in the basolateral membrane. No information about AVT action on the 36 pS Cl^- channel is available. Further, the 360 pS Cl^- may be located at the basolateral membrane after confluence, contributing to the whole cell current. No information about AVT action on the 360 pS Cl^- channel is also available. Therefore, to confirm which type of Cl^- channel contributes the basal and AVT-activated whole-cell Cl^- currents, we need more studies on Cl^- channels especially those localized in the basolateral membrane. Unfortunately, we could not identify the source of the Cl^- conductance in the present experiments. However, studies of short-circuit current have already demonstrated that ADH (including AVT) increases Cl^- secretion in A6 cells (Chalfant et al., 1993; Verrey, 1994; Doi & Marunaka, 1995) and Cl^- secretion is blocked by apically administered NPPB (Chalfant et al., 1993). Therefore, AVT-evoked Cl^- currents are likely to be composed of at least 3 pS Cl^- channels, which are sensitive to NPPB (Shintani & Marunaka, 1996), located in the apical membrane, although we cannot completely rule out the possibility that AVT also activates a basolateral Cl^- conductance.

There are at least two distinct categories of Na^+-permeant channels in epithelial cells, with varying sensitivity to amiloride or its analogues (Moran et al., 1988; Barbry et al., 1990; Smith & Benos, 1991); (i) high affinity to benzamil and amiloride (referred to as H-type; IC_{50} of amiloride $\leqslant$ 100 nM) and (ii) low affinity to benzamil and amiloride (L-type; IC_{50} of amiloride $\geqslant$ 10 μM) (Moran et al., 1988). The IC_{50} of amiloride for Na^+ channel in A6 cells is 100 nM (Eaton & Marunaka, 1990). Our preliminary observation (N. Niisato and Y. Marunaka, *unpublished data*) suggests that the IC_{50} of benzamil for the Na^+ channel is approximately 10 nM and 1 μM benzamil completely blocks the Na^+ channel. These observations suggest that the Na^+ channel in aldosterone-treated A6 cells is an H type. Benzamil has some inhibitory action on Na^+/Ca^{2+} exchanger in addition to the Na^+ channel (Kleyman & Cragoe, 1988). The IC_{50} of benzamil for the Na^+/Ca^{2+} exchanger is 100 μM (Kleyman & Cragoe, 1988). The concentration of benzamil (1 μM) used in the present study was much lower than the IC_{50} for the Na^+/Ca^{2+} exchanger. Therefore, the effect of benzamil on the Na^+/Ca^{2+} exchanger would be negligibly small. These reports suggest that the concentration of benzamil used in the present study, 1 μM, is large enough to completely block the amiloride-sensitive currents and small enough to have no significant effects on Na^+/Ca^{2+} exchanger.

Previous measurements of short-circuit current have already demonstrated that aldosterone treatment increases the amiloride-sensitive currents (Kemendy et al., 1992; Schafer & Hawk, 1992; Doi & Marunaka, 1995). Marunaka and Eaton (1991) have already reported that ADH increases the Na^+ channel number in A6 cells cultured with aldosterone. The present study has demonstrated that benzamil-blockable whole-cell currents exist in A6 cells after aldosterone treatment, although they look too small. The experimental procedures in the present study such as whole-cell configuration or EGTA treatment may have some effects on Na^+ channels and/or the sensitivity of the Na^+ channel to amiloride, resulting in small amounts of amiloride (benzamil)-sensitive currents. So, we tried to compare the amount of the benzamil-sensitive current with estimated amiloride-sensitive currents from our previous work. The benzamil-sensitive whole-cell current of about 20 pA was detected at −40 mV, which is thought to be the resting membrane potential (*see* Fig. 7*B*). Our previous study (Marunaka & Eaton, 1991) indicates that the open probability of the single amiloride-sensitive Na^+ channnel is about 0.4 at the resting membrane potential, the single channel current is about 0.3 pA and the channel density is about $2/\mu m^2$. We estimate that the area of the apical membrane is 177 μm^2 on an assumption that the cell has a columnar shape and the area of the apical membrane is equal to the cross section of the column. The diameter of columnar A6 cells used in the present study was approximately 15 μm. Since the basolateral membrane of polarized A6 cells has no amiloride-sensitive Na^+ conductance, these parameters give us the estimated value of the amiloride-sensitive current per cell; namely, it is 42 pA/cell ($=0.4 \times 0.3 \times 2 \times 177$). On the other hand, our previous report (Doi & Marunaka, 1995) indicates that the amiloride-sensitive short-circuit current is about 3 $\mu A/cm^2$; namely, it is 0.03 $pA/\mu m^2$. Therefore, the amiloride-sensitive short-circuit current/cell is estimated to be 5 pA (0.03×177). The value of the benzamil-sensitive whole cell current shown in the present study (20 pA/cell) is not too small compared with these estimated values of amiloride-sensitive current (42 pA/cell from the single channel study and 5 pA/cell from the short-circuit current study).

We were interested in the effect of AVT on the benzamil-sensitive current. Our preliminary observation suggests that AVT could not increase the benzamil-sensitive whole cell current. This is consistent with our previous observation in the short-circuit current in cells with aldosterone treatment for 3 days or more than 3 days (Doi & Marunaka, 1995). We also tried to measure the AVT action on the benzamil-sensitive whole-cell current in cells with aldosterone treatment for only one day. Unfortunately, we could not get stable whole-cell seals. So, we could not conclude whether AVT can stimulate the benzamil-sensitive whole-cell current.

We used EGTA to verify $[Ca^{2+}]_c$ in the present study. The chelating action of EGTA in response to an increase in Ca^{2+} is not fast compared with other Ca^{2+}-chelating agent such as BAPTA. The $[Ca^{2+}]_c$ adjacent to the membrane may not be acutely controlled by EGTA especially after application of AVT which might increase $[Ca^{2+}]_c$. However, the purpose of the present study was to investigate the role of the basal $[Ca^{2+}]_c$ in the basal and AVT-activated whole-cell currents. From this viewpoint, even though the $[Ca^{2+}]_c$ adjacent to the membrane was not acutely controlled by EGTA, the conclusion arrived at in the present study is useful. Further, the whole cell currents at the steady state in response to AVT show a similar dependency on $[Ca^{2+}]_c$ to the peak whole-cell current in response to AVT shown in the present study. These observations suggest that even though the $[Ca^{2+}]_c$ adjacent to the membrane cannot be acutely controlled by EGTA especially after application of AVT, the conclusion of the present study may not be changed by the buffering ability of EGTA. To investigate whether AVT acts on the whole-cell currents without any changes in $[Ca^{2+}]_c$ adjacent to the membrane, we have to use other Ca^{2+}-chelating agent such as BAPTA. Comparing the observations in the present study with those using BAPTA, we may be able to obtain the relationship between changes in $[Ca^{2+}]_c$ and the AVT action on the whole cell currents.

Besides Na^+ reabsorption, K^+ secretion is well known to be an important function of the distal nephron (Schafer, Troutman & Schlatter, 1990; Schafer, 1994). Without aldosterone treatment, Ba^{2+}-sensitive whole cell currents are very small, but Ba^{2+} shifted the reversal potential to positive potentials (Nakahari & Marunaka, 1995). Aldosterone and ADH were well known to increase K^+ secretion in the principal cells of rat cortical collecting duct (Schafer et al., 1990). Hamilton and Eaton, 1991) have demonstrated that aldosterone induces K^+-channel activity with a single-channel conductance of 13 pS, which is activated by cAMP in A6 cells. Maxi K^+ channels are also reported in the apical membrane of the mammalian cortical collecting duct (Hunter et al., 1984; Wang, Sackin & Giebisch, 1992). Two different K^+ channels, which are not activated by Ca^{2+}, are identified at the basolateral membrane of rabbit distal convoluted tubules (Taniguchi & Guggino, 1989). Although effects of Ba^{2+} on the whole-cell currents of A6 cells were unclear in the present experiments, the replacement of intracellular K^+ with Na^+ depolarized the membrane of A6 cells, suggesting that some part of the basal membrane conductance is due to K^+ channels as previously reported (Schafer et al., 1990; Schafer & Hawk, 1992; Schafer, 1994). Further, studies are needed to identify K^+ conductance in A6 cells.

At 2 nM $[Ca^{2+}]_c$, the basal currents were very small (<50 pA at a potential of 100 mV; *see* Fig. 4). The value of seal resistance was about 4 ~ 5 GΩ before breaking the patch membrane. This suggests that the seal resistance would not be larger than 4 ~ 5 GΩ after disrupting the patch membrane. If the seal resistance is 5 GΩ, the leak current at a potential of 100 mV should be 20 pA, which is almost identical to the level of the basal current in 2 nM $[Ca^{2+}]_c$. The estimated leak current of 20 pA is the minimum value; i.e., the leak current is ⩾20 pA at 100 mV in 2 nM $[Ca^{2+}]_c$. This observation suggests that most of the basal current in 2 nM $[Ca^{2+}]_c$ is due to leak current.

In conclusion, the basal and ADH-induced conductances in aldosterone-treated A6 cells require a minimum level of intracellular calcium (about 30 nM), and a reduction to lower levels leads to no measurable membrane conductance.

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