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Mechanism of Aldosterone-Induced Increase of K^+ Conductance in Early Distal Renal Tubule Cells of the Frog

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Summary. Isolated early distal tubule cells (EDC) of frog kidney were incubated for 20–28 hr in the presence of aldosterone and then whole-cell K^+ currents were measured at constant intracellular pH by the whole-cell voltage-clamp technique. Aldosterone increased barium-inhibitable whole-cell K^+ conductance (gK^+) threefold. This effect was reduced by amiloride and totally abolished by ouabain. However, aldosterone could still raise gK^+ in ouabain-treated cells in the presence of furosemide.

We tested whether changes in intracellular pH (pH_i) could be a signal for cells to regulate gK^+. After removal of aldosterone, the increase in gK^+ was preserved by subsequent incubation for 8 hr at pH 7.6 but abolished at pH 6.6. In the complete absence of aldosterone, incubation of cells at pH 8.0 for 20–28 hr raised pH_i and doubled gK^+.

Using the patch-clamp technique, three types of K^+-selective channels were identified, which had conductances of 24, 45 and 59 pS.

Aldosterone had no effect on the conductance or open probability (P_o) of any of the three types of channels. However, the incidence of observing type II channels was increased from 4 to 22%. Type II channels were also found to be pH sensitive, P_o was increased by raising pH.

These results indicate that prolonged aldosterone treatment raises pH_i and increases gK^+ by promoting insertion of K^+ channels into the cell membrane. Channel insertion is itself triggered by raising both pH_i and increasing the activity of the Na^+/K^+ pump in early distal cells of frog kidney.

Key Words K^+ channel · intracellular pH · Na^+-K^+ ATPase · patch-clamp · amphibian kidney · aldosterone

Introduction

The classical renal effects of aldosterone are an increase of sodium reabsorption and of potassium secretion by the distal nephron of the mammalian kidney [4, 5]. The mechanisms of the stimulatory effects of aldosterone on sodium reabsorption and potassium secretion involve increases in luminal Na^+ permeability, the activity of the Na^+-K^+ pump and the cellular K^+ permeability [8, 23, 25, 36, 40]. This latter effect is believed to be a direct effect of mineralocorticoid hormone action [40].

In the amphibian kidney, adaptation to a high potassium environment (a state that leads to an increase in plasma aldosterone level) causes secretion of K^+ and H^+ in the early distal tubule [14, 28, 31]. Amiloride inhibits aldosterone-induced H^+ secretion [46]. Stimulation of luminal Na^+/H^+ exchange increases K^+ secretion, whereas inhibition reduces K^+ secretion [26, 32]. It has been reported that acute administration of aldosterone (2 hr), dramatically increases intracellular pH in fused giant cells of the early distal tubule of frog kidney [34]. In the same preparation, a pH-sensitive K^+ conductance has been observed [21, 29]. This raises the possibility that luminal Na^+/H^+ exchange could be one target of aldosterone whose action is to elevate intracellular pH and increase luminal K^+ conductance.

The present experiments were designed to test this hypothesis further, and to extend the period of observation (20–28 hr) of aldosterone's action on both cell pH and K^+ conductance. To study the effect of aldosterone on cell K^+ conductance, we employed the whole-cell voltage-clamp technique. By dialyzing the cell with a solution of constant pH, we avoided changes in cell conductance due to acute alterations in pH, and so were able to investigate the effects of prior treatment protocols on whole-cell K^+ conductance. We used the single-channel patch-clamp method to identify the different types of K^+ channels involved in the response of the cell to aldosterone.

We demonstrate that, in contrast to acute exposure to aldosterone [34], longer exposure to the hormone produces only a moderate elevation of intra-

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cellular pH, but, nevertheless, this is accompanied by a large increase in whole-cell K^+ conductance, which occurs through an effective recruitment of K^+ channels into the cell membrane. While a rise in intracellular pH augments K^+ conductance, cytoplasmic acidification has the opposite effect.

Our observations support the hypothesis [34] that one of the mechanisms by which aldosterone modulates the K^+ conductance of early distal tubule cells of frog kidney is by stimulation of Na^+/H^+ exchange.

Materials and Methods

Isolation of Single Cells

Early distal tubule cells were isolated from the kidneys of *Rana pipiens*. A detailed account of this technique has been published previously [33]. Briefly, frog kidneys were first perfused via the aorta with a Ca^{2+} and Mg^{2+}-free Ringer solution (50 ml) composed of (in mM): 95 NaCl; 3 KCl and 10 HEPES: pH 7.60 (with NaOH). Then, the kidneys were perfused (10 ml) via the portal veins with Ringer's solution containing 1.0 mM Ca^{2+}, 1.0 mM Mg^{2+} and collagenase (0.05%, 180 units/mg; Sigma, St. Louis, MO). Small pieces of the ventral surface containing primarily diluting segments were removed with fine scissors and incubated for 45 min at 25°C in 2 ml of the collagenase-containing kidney perfusate. Then, single cells were isolated by gently sucking the tubular fragments up and down with a pasteur pipette. The isolated cells were transferred into a conical tube and centrifuged ($400 \times g$, 3 min). The supernatant was removed and the cells were resuspended in Ringer's solution containing (in mM) NaCl 95; KCl 3; $MgCl_2$ 1; $CaCl_2$ 1; HEPES 10 and glucose 5, pH 7.60 (with NaOH). Finally, cells were transferred onto thin glass cover slips that were pretreated with poly-1-lysine (0.1 g/liter; Serva).

Cells were first incubated in different solutions for 20–28 hr according to the experimental protocol, then transferred to a chamber for whole-cell or patch-clamp study. Some of the cells were incubated in aldosterone (1 μM, Sigma, St. Louis, MO)-containing Ringer's solution. In other suspensions 0.1 mM amiloride (Merck, Rahway, NJ), 0.1 mM ouabain (Sigma) and 0.05 mM furosemide (Sigma) were added. The pH of the incubation solution was usually kept at 7.60. However, during some experiments the pH of the incubation solutions was set to 8.00 or 6.60. Cell viability was assessed by Trypan blue exclusion and was usually greater than 80%. However, following incubation in ouabain-containing solution, there was a reduction in viability to less than 30%.

Patch and Whole-Cell Voltage-Clamp Techniques

Patch and whole-cell voltage-clamp experiments were performed according to the method of Hamill et al. [15]. Patch-clamp electrodes were fabricated from glass capillaries (Drummond Scientific) with resistances of 2 to 5 MΩ when filled with saline. Recordings were made using a List L/M-EPC7 patch-clamp amplifier (List Medical, Darmstadt, FRG). For measurement of whole-cell currents, 60 sec were allowed for equilibration of the cell with the pipette solution. In separate experiments (*not presented*) dye transfer from pipette to cell was found to be complete within 1 min. Single-channel currents were low pass filtered at 1 kHz using an eight-pole Bessel filter (902LPF: Frequency Devices, Haverhill MA), digitized at a sampling rate of 44 kHz using a modified Sony PCM-501ES pulse code modulator and stored on videotape (Sony SL-2700). Single-channel data was analyzed using the *p*Clamp software system (Version 4.05; Axon Instruments, Burlingame, CA). Channel conductance was calculated by recording single-channel currents at a number of different holding potentials. Open probability (P_o) of individual single K^+ channels was determined as follows:

$$P_o = (1/N) \cdot \Sigma(t_1 + t_2 \ldots \ldots t_n) \quad (1)$$

where N was the number of channels, i.e., the maximum numbers of superpositions of current level seen in the patch, and t the fractional open time spent at each of the observed current levels.

Single-channel incidence was assessed as the percentage of patches containing a particular channel type in patches that were successful, i.e., patches with seal resistances >10 GΩ.

Whole-cell currents were low pass filtered at 1.5 kHz and sampled directly onto a computer hard disk (IBM XT 286).

Solutions for Patch-Clamp Study

Table 1 shows the composition of the bath solution for measuring whole-cell and single-channel currents. Solution 1 was used to measure both whole-cell and single-channel K^+ currents. Solutions 1 and 2 were used to evaluate the selectivity of K^+ over Na^+ of single-channels. Solutions 3 and 4 were applied to study the effect of pH on channel activity. Solution 1 was replaced by solution 5 to investigate the sensitivity of the channel to Ca^{2+}. The pipette solution contained (in mM) K gluconate 80; KCl 10; $MgCl_2$ 1.0; EGTA 5.0; HEPES 10; pH 7.3 (with KOH). All solutions were filtered prior to use (0.22 μm, Millipore, Bedford, MA). Where appropriate, barium (as $BaCl_2$) was added to the bath solution to achieve a final concentration of 2 mM.

Measurement of Intracellular pH

Fluorescence Method

The pH-sensitive dye BCECF ([2′,7′,-bis(2-carboxyethyl)-5 (and 6) carboxyfluorescein]), has a peak excitation near 490 nm that is sensitive to pH, and an isosbestic point at 436 nm, which is pH insensitive. The peak emission is at 536 nm [1, 2]. Therefore, for this series of experiments the cells were excited at 440 and 490 nm, respectively, and the emission was monitored at 536 nm. After correcting all measurements for background, the mean of the 490-nm excitation was divided by the 440-nm excitation measurement, yielding the fluorescence excitation ratio (F_{490}/F_{440}).

Isolated cells were viewed on the stage of an inverting microscope (Zeiss IM35) at 630× magnification. Cells were loaded with dye by incubation at 20°C for 15 min in the presence of 10 μM acetoxymethyl ester of BCECF (BCECF-AM). After loading, the intracellular dye was excited by exposing each cell for 200 msec at 490 nm, and then for 200 msec at 440 nm. The time between excitation at the individual wavelengths was 10 msec and there was a 2-sec pause between excitation sample pairs.

Table 1. The composition of the bath solutions

	K^+	Na^+	Mg^{2+}	Ca^{2+}	Gluconate (in mM)	Cl^-	EGTA	HEPES	pH
1.	98	0	1	1	90	12	0	10	7.5
2.	30	68	1	1	90	12	0	10	7.5
3.	98	0	0	0	90	8	0	10	8.0
4.	98	0	0	0	90	8	0	10	7.2
5.	98	0	1	0	90	10	5	10	7.5

The fluorescence excitation data for each wavelength was recorded for each of the cells at the pH of the incubation solutions.

Data Acquisition and Detection System

We used a detection system similar to that recently described by Boyarsky et al. [2] with minor modifications. Briefly, the emission data for each of the wavelengths was detected by a photomultiplier tube (PMT). The output of the PMT was sampled through a headstage preamplifier and an integration circuit. The digitized output (from the integration circuit) was stored on the hard disk of a PDP 11/73 computer and displayed as either the output at the individual wavelengths or as a fluorescence excitation ratio of F_{490}/F_{440}.

Nigericin-pH Calibration Technique

Cells were loaded with 50 μM nigericin solution for 10 min and then exposed to solutions of different pH, as first described by Thomas et al. [41]. Calibration curves were obtained and used to calculate the steady-state pH of each cell.

Statistics

Data are presented as means ± SEM. Differences between groups were assessed by means of the Student's *t*-test for unpaired data *P* values of <0.05 were taken as statistically significant.

Results

Whole-Cell Conductances of Single Early Distal Cells

Figure 1 shows representative current-voltage curves recorded from an early distal tubule cell in response to clamping the membrane potential to positive and negative values. The whole cell currents are inwardly rectifying, as evidenced by the outward slope conductance (calculated from the positive currents) being smaller than the inward slope conductance (calculated from the negative currents between -100 and -50 mV). Ba^{2+} (2 mM) in the bath largely inhibited both the inward and outward currents. In the following text only the barium-sensitive component is referred to as K^+ conductance (gK^+). The control gK^+ of cells was measured after 20–28 hr incubation. The average inward gK^+ in the physiological range of cell membrane potentials at an intracellular pH of 7.3 was 4.2 ± 0.7 nS ($n = 9$), the average outward gK^+ was 2.3 ± 0.3 nS (Table 2).

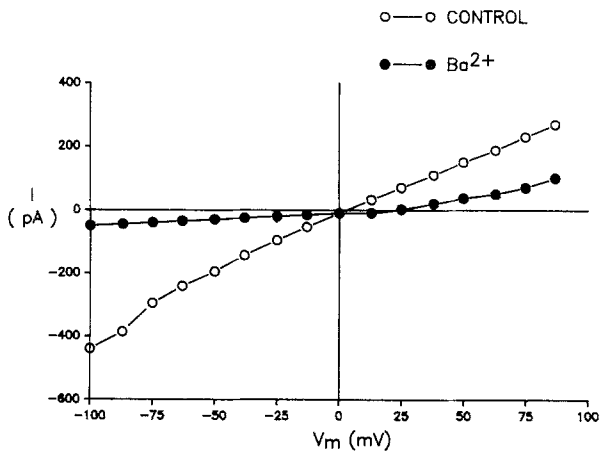


Fig. 1. Current-voltage curves of a single early distal cell under control conditions (open circles) and in the presence of 2 mM Ba^{2+} (filled circles). Membrane potentials were clamped to ±100 mV in steps of 12.5 mV. The pipette solution contained 90 mM K-gluconate solution, and the bath solution 98 mM K-gluconate (solution 1)

Effect of Aldosterone on gK and pH_i

Incubation of cells in medium containing aldosterone (1 μM) for 20 hr increased significantly both the inward and outward gK^+ (Table 2). Previous observations in fused early distal cells of frog kidney showed that acute application of aldosterone stimulates Na^+/H^+ exchange and increases intracellular pH [32]. This study confirms these earlier observations. After 20 hr incubation of cells in aldosterone-containing solution intracellular pH was significantly higher (7.55) than the control value (7.43, Table 3). However, in contrast to acute application

Table 2. Effect of aldosterone on Ba^{2+}-sensitive K^+ conductance (gK^+)

	Control	Aldosterone
Inward gK^+ (nS)	4.2 ± 0.7	12.5 ± 0.7[a]
Outward gK^+ (nS)	2.3 ± 0.3	7.7 ± 0.9[a]
N	9	10

Values are mean ± SEM; N is the number of measurements. The values for gK^+ are the slope conductance.
[a] $P < 0.05$.

Table 3. The effects of aldosterone and changing extracellular pH (pH_o) on intracellular pH (pH_i)

	Control	Aldosterone	pH_o 8.00	pH_o 6.60
pH_i	7.43 ± 0.04	7.55 ± 0.04[a]	7.70 ± 0.03[a]	7.10 ± 0.04[a]
N	8	7	7	8

The control pH_i was measured after cells were incubated in normal Ringer solution (pH 7.60) for 20–28 hr. The pH of aldosterone-treated cells was measured in aldosterone-containing solution (pH 7.60) after incubation for 20–28 hr. pH_i in the group of pH_o 8.00 was obtained after cells were incubated in pH 8.00 Ringer for 28 hr. pH_i in the fourth group (pH_o 6.60) was measured after cells were first in pH 7.60 aldosterone-containing solution for 20 hr and then changed to aldosterone-free pH 6.60 medium for another 8 hr. Values are mean ± SEM; N is the number of cells.
[a] $P < 0.05$.

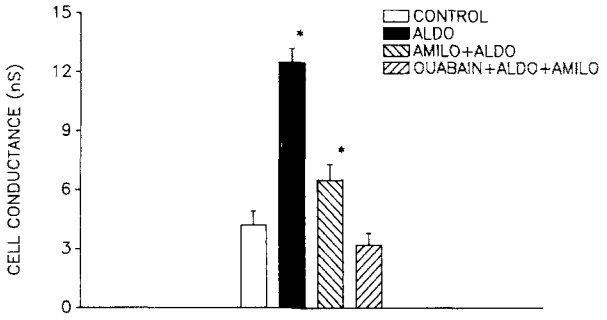


Fig. 2. The effect of aldosterone on gK^+ in the presence of amiloride and ouabain. Cells in the control group were first incubated in the solution containing (in mM) 95 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 5 glucose, pH 7.60, for 20–28 hr. The cells in the other groups were incubated in the same solution containing either 1 μM aldosterone (*ALDO*), 1 μM aldosterone + 0.1 mM amiloride (*AMILO + ALDO*), or 1 μM aldosterone + 0.1 mM ouabain + 0.1 mM amiloride (*OUABAIN + ALDO + AMILO*) for 20 h. After incubation the cells were transferred to a chamber to measure the whole cell K^+ current. The composition of solutions in the bath and the pipette was as for Fig. 1. (* = $P <$ 0.05)

[32], long-term exposure to aldosterone produces a more moderate alteration in intracellular pH.

To test the hypothesis that the aldosterone-induced increase in pH_i directly raises gK^+, we tested the effects of 0.1 mM amiloride (a known inhibitor of Na^+/H^+ exchange in various cell types [18, 31]), on the aldosterone-induced increase in gK^+. In the presence of amiloride, gK^+ was significantly lower (6.5 ± 0.8 nS, $n = 9$; Fig. 2) than that (12.5 ± 0.7 nS; $n = 10$) in the absence of amiloride, but was still higher than the control value. Because the concentration of amiloride used has been shown to maximally inhibit Na^+/H^+ [31], these results suggest that aldosterone increases gK^+ not only by increasing pH_i, but also by an additional mechanism, possibly through stimulation of Na^+-K^+-ATPase activity [17]. We investigated this by using ouabain to inhibit the enzyme. Figure 2 includes experiments in which ouabain (0.1 mM) was used in combination with 0.1 mM amiloride. We note that the aldosterone-induced increase of gK^+ was totally abolished (3.2 ± 0.6 nS; $n = 8$). Amiloride is also known to inhibit Na^+-K^+ ATPase in addition to inhibiting Na^+/H^+ exchange [39]. Therefore, it could be argued that the effect of amiloride was solely due to partial inhibition of the Na^+-K^+ ATPase. Accordingly, efforts were made to exclude this possibility.

We examined whether aldosterone could still increase gK^+ while the pump was inhibited. Figure 3 shows that when cells were incubated in medium containing aldosterone and ouabain (0.1 mM) for 20 hr, gK^+ was 3.1 ± 0.8 nS ($n = 5$). This was not statistically different from cells incubated under control conditions and was similar to the gK^+ of cells incubated in ouabain containing solution without aldosterone (2.8 ± 0.7 nS; $n = 6$, Fig. 3). This, at first, would suggest that aldosterone's effects on gK^+ are mediated entirely via its effects on the Na^+-K^+ ATPase. However, during inhibition of the pump by ouabain, intracellular Na^+ rises and the gradient for Na^+/H^+ exchange is dissipated [45]. This would prevent the aldosterone-induced increase in intracellular pH and therefore, any pH-dependent effects on gK^+. In order to investigate this further, we incubated cells in furosemide, to maintain the sodium gradient following inhibition of Na^+-K^+ ATPase (*see* Discussion). Figure 3 shows that in the presence of 0.05 mM furosemide, aldosterone significantly increased gK^+ (6.8 ± 1.0 nS; $n = 8$) even after inhibition of Na^+-K^+ ATPase by ouabain. In contrast, incubation of cells in the absence of aldosterone, but in the presence of furosemide and oubain had no effect on gK^+ (2.8 ± 0.3 nS; $n = 5$).

From this series of experiments, we conclude that aldosterone is able to stimulate Na^+/H^+ and

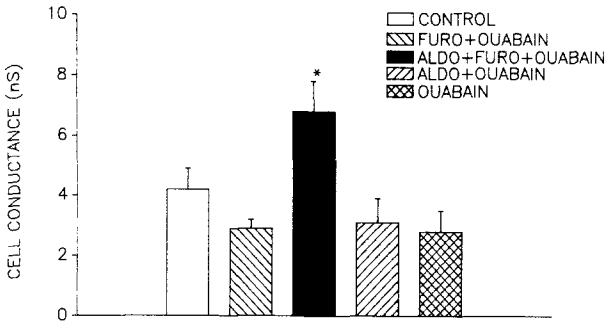


Fig. 3. The effect of aldosterone on gK^+ during inhibition of Na^+-K^+ ATPase. Cells of the control group were incubated for 20–28 hr in the same solution described in Fig. 2. Cells of the other groups were incubated in the same solution containing 0.05 mM furosemide + 0.1 mM ouabain (*FURO + OUABAIN*); 1 μM aldosterone + 0.05 mM furosemide + 0.1 mM ouabain (*ALDO + FURO + OUABAIN*); 1 μM aldosterone + 0.1 mM ouabain (*ALDO + OUABAIN*) or 0.1 mM ouabain (*OUABAIN*). After incubation, gK^+ was measured as described for Fig. 2. The composition of the solutions was as described for Fig. 1 (* = $P < 0.05$)

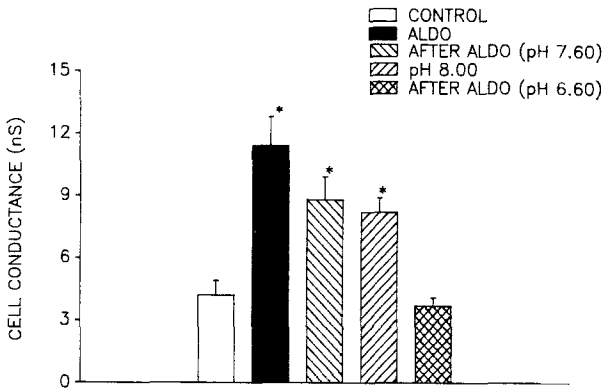
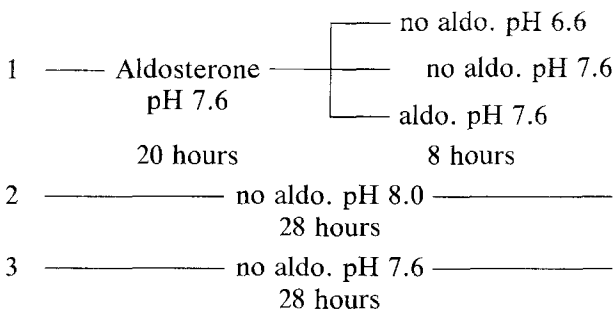


Fig. 4. Effect of pH on gK^+. Cells of the control and ALDO groups were incubated as described for Fig. 2. The cells in the remaining groups were subsequently transferred to aldosterone-free solution at either pH 7.60 or 6.60 for another 8 hr as described in the text. The cells in the pH 8.00 group were incubated in aldosterone-free pH 8.00 medium for 28 hr. After incubation all K^+ currents were measured with solutions as described for Fig. 1. (* = $P < 0.05$)

raise cell gK^+ even after inhibition of Na^+-K^+ ATPase by ouabain. However, aldosterone must have a dual effect, since the effect on gK^+ was reduced by ouabain.

Relationship between Intracellular pH and gK^+

Aldosterone can stimulate Na^+/H^+ exchange and raise intracellular pH within two hours of application [34]. We carried out two types of experiments: (i) To measure pH_i after chronic (20–28 hr) exposure to aldosterone, and (ii) to investigate the effects of cell pH on gK^+. As stated earlier, we found that aldosterone increased pH_i (Table 3); however, the magnitude of the rise in pH was smaller than that produced by acute application [34]. Since amiloride partly abolished the aldosterone-induced increase of gK^+ (Fig. 2), we tested whether cell pH could be a signal to regulate gK^+. Three groups of cells (1, 2, and 3) were incubated as shown in the flow-diagram below:



When cells were incubated in aldosterone-free solution at pH 8.0 for 28 hr, both intracellular pH and gK^+ were raised (Fig. 4 and Table 3). Although the rise in intracellular pH was greater than that caused by aldosterone, the increase in gK^+ produced by the alkalosis was smaller. These observations are again consistent with the idea that aldosterone has dual mechanisms of increasing potassium conductance.

Incubation of cells in aldosterone-containing solution for 28 hr raised both pH_i (Table 3) and gK^+ (12.0 ± 0.7 nS; Fig. 4). Changing to pH 6.60 aldosterone-free solution for 8 hr (after incubating in aldosterone-containing solution for 20 hr) reduced pH_i (Table 3), and the aldosterone-induced increase in gK^+ was abolished (3.7 ± 0.4 nS; Fig. 4). In contrast, incubation of cells in aldosterone-free solution pH 7.6 for 8 hr following removal of aldosterone maintained the enlargement of gK^+ (8.1 ± 1.1 nS; Fig. 4).

The inhibition of aldosterone's effect on gK^+ by intracellular acidosis leads us to conclude that changes in intracellular pH can act as a signal for the insertion in, or withdrawal of, K^+ channels from the cell membrane.

Single-Channel Conductances

Single-channel currents were measured in the cell-attached mode with 98 mM K^+ in the bath and 90 mM K^+ in the pipette. Three types of K^+ channels were observed in the control and aldosterone-treated groups (Table 4). All of the three types of

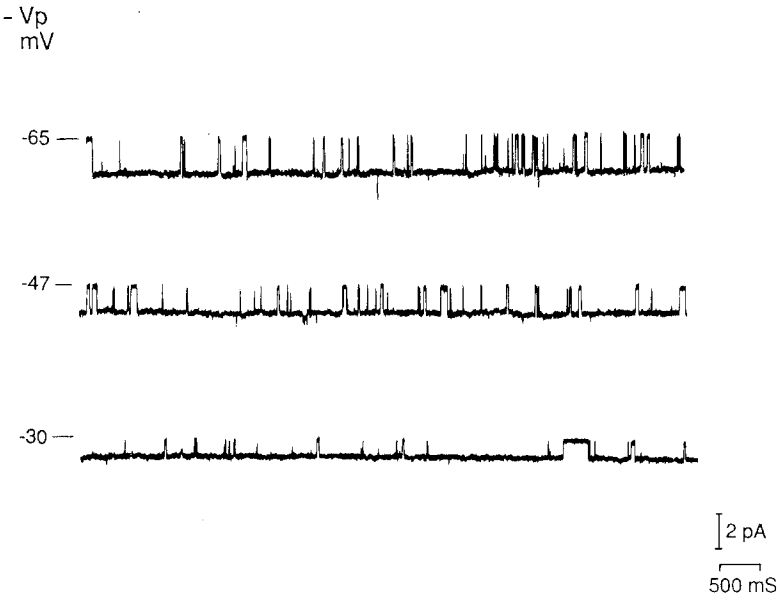


Fig. 5. A recording of a type I K^+ channel made in the cell-attached mode. The bath solution was (in mM): 90 K-gluconate, 8 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, pH 7.50. The pipette solution was (in mM): 80 K-gluconate, 10 KCl, 1 $MgCl_2$, 5 EGTA and 10 HEPES, pH 7.30. Inward currents are displayed as downward deflections. The closed state is indicated by the short bar

Table 4. The conductance of K^+ channels of EDC of frog kidney

	Inward (pS)	Outward (pS)	*N*
Type I	24.4 ± 0.7	no detectable	7
Type II	44.8 ± 0.6	21.4 ± 0.8	10
Type III	58.8 ± 1.7	34.4 ± 1.7	10

Values are mean ± SEM; *N* is the number of measurement. The conductance of the channels is the slope conductance.

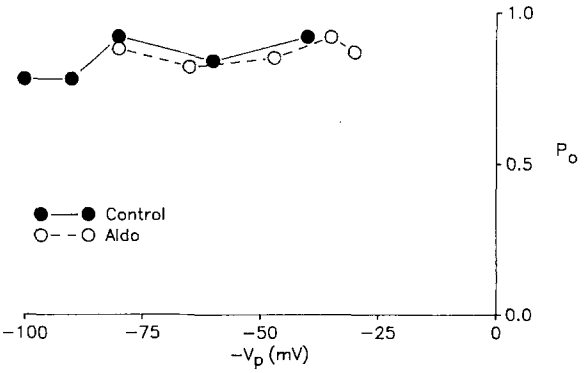


Fig. 6. Open probability of type I channels in control (filled circle) and aldosterone-treated cells (open circle). $-V_p$ is the cell membrane potential with respect to the patch pipette at ground

channel in inside-out patches were inhibited by addition of barium to the bath solution (data only presented for type II channels: *see below*).

Figure 5 shows the first type of K^+ channel (type I). Mean single-channel inward conductance was approximately 24 pS, but no outward current could be detected. The open probability (P_o) of type I channels was not voltage dependent (Fig. 6).

The mean inward conductance of the second type of channel (type II) was approximately 45 pS and the outward conductance was 21 pS (Table 4; Fig. 7). The channel open probability was voltage dependent, such that depolarization of the cell membrane potential increased P_o (Fig. 8*a*).

Figure 9 shows the third type of K^+ channel (type III). The inward conductance was approximately 59 pS and the outward conductance was 34 pS. The channel opening was also voltage dependent; membrane depolarization raised P_o (Fig. 8*b*).

Incubation of cells in aldosterone-containing solution for 20 hr did not significantly alter either single-channel conductance or open probability of any of the three types of channel (Table 5, Fig. 6 and 8*a* and *b*). However, the incidence with which we observed the type II channel was increased significantly: We observe type II channels in only 4% of patches from cells under control conditions but in 22% of patches from aldosterone-treated cells in which adequate seals were obtained (Table 5).

Further Characterization of the 45-pS K^+ Channel (Type II)

The results for ten experiments using cell-attached patches are summarized in Fig. 10. The channels showed marked inward rectification. The intracellular K^+ activity of amphibian early distal cells has been shown to be between 50 and 60 mM [28]. Since the pipette K^+ concentration was similar to that of the cell (assuming an activity coefficient of 0.76), this rectification could not result from differences in

Table 5. Single-channel conductance and incidence in control and aldosterone-treated cells

	Control			Aldosterone		
	Conductance (pS)	*N*/seal	%	Conductance (pS)	*N*/seal	%
Type I	24 ± 0.7	4/51	7.8	25 ± 0.8	3/36	8.3
Type II	44	2/51	3.9	45 ± 0.6	8/36	22.2[a]
Type III	58 ± 1.7	6/51	11.7	60 ± 2.0	4/36	11.1

Values are mean ± SEM; *N* is observed number of channels. Seal means all patches that seal resistance was over 10 GΩ.
[a] $P < 0.05$ by Chi-squared test.

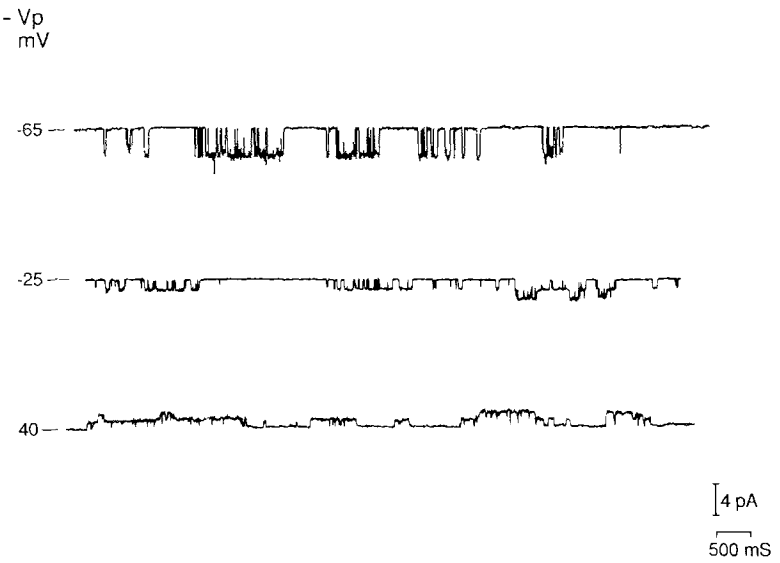


Fig. 7. K^+ currents of a type II channel recorded from a cell-attached patch. The compositions of the bath and the pipette solution were as in Fig. 5. Inward currents are displayed as downward deflections. The closed state is indicated by the short bar

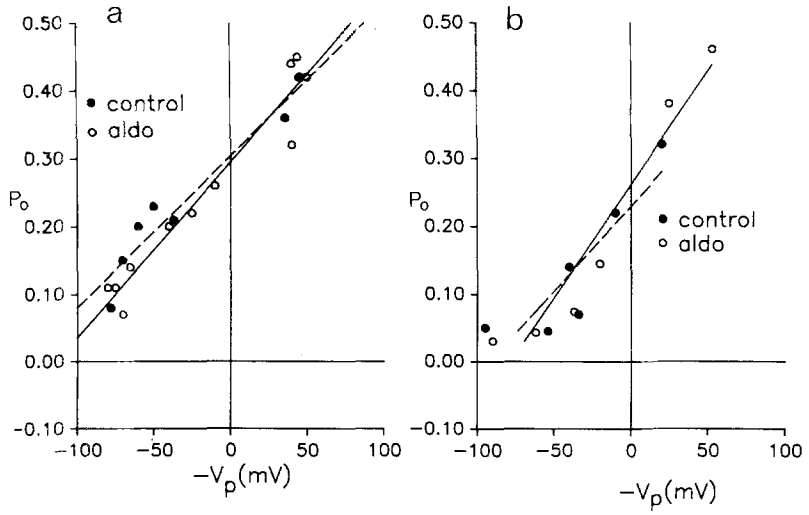


Fig. 8. (*a*) The relationship of cell voltage ($-V_p$) and channel open probability (P_o) of type II and (*b*) type III channels. There is no significant difference between the slope of the regression lines for either the control (-----) or aldosterone treated (—) cells

ion concentration between the intra and extracellular solutions (Goldman rectification).

These channels were not calcium activated, since channel activity in inside-out patches was not different either in the absence, or in the presence of 1 mM calcium in the bath solution (*data not shown*).

The selectivity of the channels to Na^+ was studied using inside-out patches. Figure 11 shows that when 60 mM potassium gluconate and 8 mM KCl in the bath were substituted by equimolar amounts of the sodium salts (leaving 30 mM potassium in the bath). For three such experiments, the mean rever-

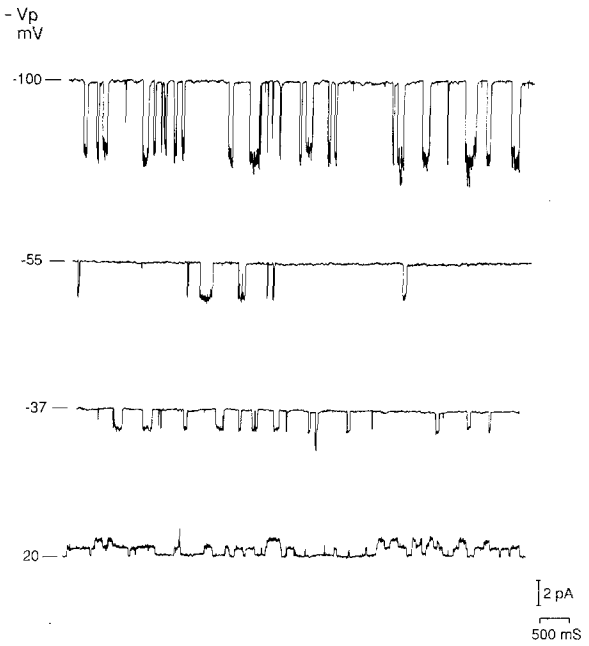


Fig. 9. Single-channel K^+ currents of the type III channel recorded from a cell-attached patch. The solutions for the bath and pipette were the same as described for Fig. 5. Inward currents and displayed as downward deflections. The close state is indicated by the short bar

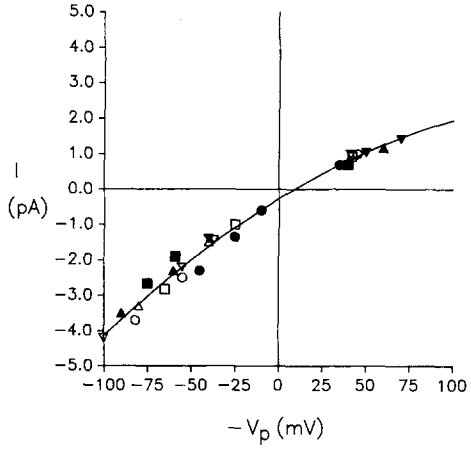


Fig. 10. Current-voltage relationship for ten individual type II channels. Recordings were made in the cell-attached configuration with solutions as described in Fig. 5. The solid line is a second order polynomial regression

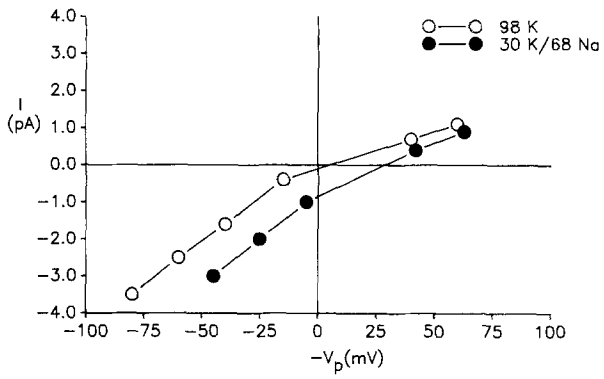


Fig. 11. Current-voltage relationships for a type II K^+ channel in an inside-out patch. The pipette solution contained (in mM): 80 K-gluconate, 10 KCl, 1 $MgCl_2$, 5 EGTA and 10 HEPES, pH 7.30. The bath contained either 90 K-gluconate, 8 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES, pH 7.50 (open circles); or 60 Na-gluconate, 30 K-gluconate, 8 NaCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES, pH 7.50 (filled circles)

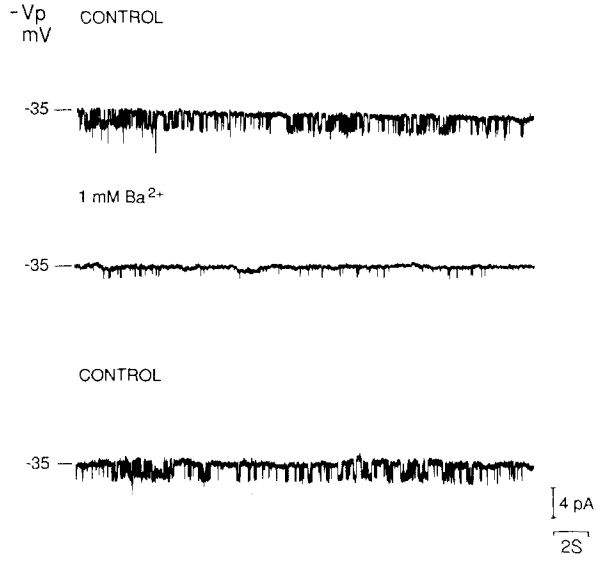


Fig. 12. Effect of Ba^{2+} on a type II channel in an inside-out patch. The pipette solution was as described in Fig. 11. The bath contained (in mM): 90 K-gluconate, 8 KCl and 10 HEPES, pH 8.00. Barium was added to the bath to reach a final concentration of 1 mM. The effect of barium on channel activity was reversible. After barium was washed out (control), the channel activity was restored. The closed state is indicated by the short bar

sal potential shifted by 23.6 ± 2.0 mV (which is close to that expected for a K^+-selective channel). The selectivity ratio of the channel (P_K/P_{Na}) was determined using the equation

$$E_{rev} = 58 \cdot \log[K_b + (P_{Na}/P_K) \cdot Na_b]/K_p \qquad (2)$$

where K_b, Na_b and K_p are the concentrations of K^+ and Na^+ in the bath and K^+ in the pipette, respectively. From these experiments a K^+/Na^+ selectivity for the channel of 8.1 : 1 was calculated.

Type II channels were reversibly inhibited by barium (Fig. 12). Barium applied in the bath solution significantly reduced the channel open probability in inside-out patches, but had no effect on single-channel conductance.

When the pH of the bath solution was lowered, type II channel activity in inside-out patches was

sharply reduced (Fig. 13*a*). Figure 13*b* is a summary of two such experiments. When the pH of the bath solution (facing the cytoplasmic surface of the membrane) was raised from 7.20 to 8.00, the curve of voltage-dependent channel opening was shifted to the left. In the physiological range of membrane potentials, changing pH from 7.2 to 8.0 doubled the open probability. Since the single-channel conductance was not altered, the effective K^+ current will increase twofold. These observations are in good agreement with previous studies, which have shown a pH-dependent K^+ conductance in this cell type [21].

Discussion

Whole-Cell Conductance under Control Conditions

Figure 14 shows a schematic view of the early distal cell of frog kidney. The cell contains both apical and basolateral K^+ conductances. It is known that under normal circumstances K^+ secretion does not occur [28]. Only if the animal is adapted to a high K^+ environment, and the plasma aldosterone level rises, does this segment secrete K^+.

Separate studies [21, 29, 34] have shown that aldosterone increases intracellular pH in early distal cells and that this alkalinization is accompanied by a rise in the K^+ permeability of the cell membrane. The enhancement of K^+ permeability could be due to activation of existing K^+ channels (by raising open probability), insertion of extra K^+ channels in the membrane or both mechanisms. These possibilities cannot be distinguished solely by macroscopic conductance measurements. For this reason, we used the patch-clamp technique to identify single channels and the whole-cell voltage-clamp method to measure K^+ conductance. The latter technique has the advantage that the cell becomes completely dialyzed by the solution within the pipette, so by choosing a suitable pipette solution it is possible to select the type of currents to be studied, and to precisely control intracellular composition.

Previous experiments have shown the presence of calcium-sensitive "maxi" K^+ channels in the apical membranes of the *Amphiuma* early distal tubule cells [19]. However, in the physiological range of cell membrane potentials the calcium-activated channel contributes little to the apical membrane potassium conductance. In the present experiments, we have studied only the calcium-insensitive component of the whole-cell potassium conductance (gK^+). The inward gK^+ (4.2 nS) found in this study is lower than one (12.9 nS) measured in an earlier study [21]. The discrepancy can be explained

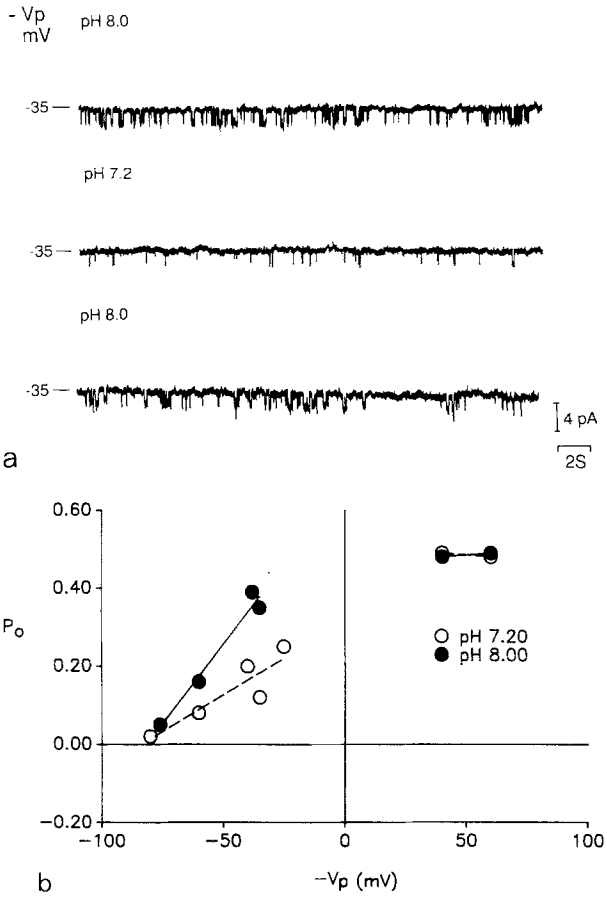


Fig. 13. (*a*) The effect of pH on K^+ currents of a type II channel recorded in an inside-out patch. The pipette solution was as described in Fig. 11. The bath solution was composed of (in mM): 90 K-gluconate, 8 KCl and 10 HEPES; pH was adjusted to 8.00 or 7.20. The closed level is indicated by the short bar. The channel activity was reduced when the bath pH was lowered. (*b*) The effect of pH on open probability for two individual type II channels

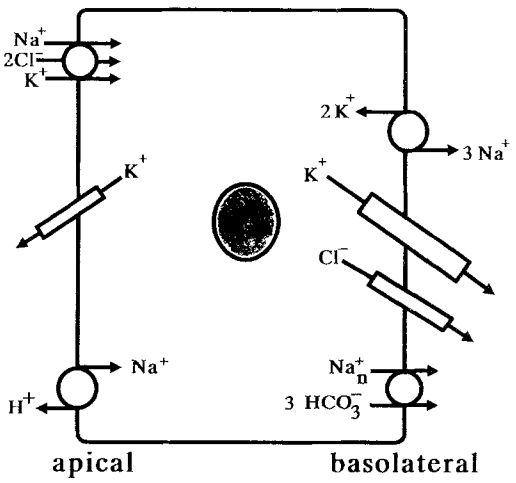


Fig. 14. Cell scheme of early distal tubule of amphibian kidney under control conditions. Normally cellular K^+ movement occurs in the absorptive direction

by different intrapipette (intracellular) pH (present 7.30, previous 7.60–8.00). The outward gK^+ (2.3 nS) of this study is close to the value of 1.5 nS measured previously. This suggests that the inhibition of gK^+ by H^+ is voltage dependent. Our studies on type II channels (*see below*) support this idea.

Aldosterone Increases the Number of K^+ Channels

After incubating cells in aldosterone-containing medium, gK^+ rose nearly threefold. This aldosterone-induced augmentation of gK^+ could result from an increase of total single-channel current. However, we have shown that long term aldosterone treatment did not change the conductance or open probability of any of the thee types of channels. During whole-cell recording, intracellular pH was held constant and any intracellular factor that might increase the single-channel current would presumably be removed after the cell was dialyzed by the pipette solution. Accordingly, the aldosterone-induced augmentation of whole-cell K^+ current must result from an elevation in channel density. The rise in the incidence of the 45-pS K^+ channel after aldosterone incubation further supports this conclusion. However, the pH dependence of type II channels that we have demonstrated suggests that changes in single-channel current may be the basis of acute changes in gK^+ during larger elevations of intracellular pH, as occurs during the early phase of aldosterone action [32].

Aldosterone is known to activate recruitment of Na^+ channels into membranes. In the toad urinary bladder the aldosterone-dependent increase of sodium permeability is a result of an equivalent elevation in the density of conducting apical sodium channels [36]. The present study shows that aldosterone can also trigger recruitment of K^+ channels. Whether this recruitment is due to synthesis and insertion of new channels or activation of pre-existing "silent" channels is not known.

Aldosterone Stimulates the Na^+/H^+ Exchanger

Amiloride is a known blocker of the epithelial sodium channel at micromolar concentrations [16, 35]. At millimolar concentrations, amiloride also inhibits sodium-hydrogen exchange [18, 32]. Since no sodium conductance is present in the frog early distal cell, the antagonistic effect of amiloride on the aldosterone-induced increase of gK^+ suggests that the effect of aldosterone on the gK^+ of early distal tubule cells of frog kidney results, at least partially, from stimulation of sodium-hydrogen exchange. Because amiloride can inhibit Na^+-K^+ ATPase [39], it might be argued that the effect of amiloride on the aldosterone-induced increase in gK^+ could be due to inhibition of Na^+-K^+ ATPase, which then indirectly decreases gK^+. Indeed, the observation that in the presence of ouabain, aldosterone failed to produce any increase of gK^+ seems at first, to suggest that the aldosterone-induced increase of gK^+ results solely from increasing activity of the Na^+-K^+ ATPase. However, under such conditions ouabain increases the intracellular sodium activity [45] and reduces the sodium gradient that energizes the Na^+/H^+ exchanger. Thus, ouabain indirectly inhibits Na^+/H^+ exchange. Using furosemide in combination with aldosterone and ouabain confirms this hypothesis. Furosemide, a loop diuretic, inhibits $Na^+/2Cl^-/K^+$ cotransport and abolishes the sodium influx through this carrier, which is the main pathway for Na^+ entry in this cell type [27]. Under such conditions, inhibition of the Na^+-K^+ ATPase has less effect on the intracellular electrolyte activities, and the gradients favoring Na^+/H^+ exchange are maintained. This hypothesis is supported by the findings of several studies [10, 11, 13]: In the diluting segment of *Amphiuma* kidney, the addition of oubain after furosemide inhibition does not increase cell volume [13]. In the thick ascending limb of Henle's loop of rabbit kidney, furosemide prevents cells from depolarization induced by removal of metabolic substrates [10, 11]. However, the aldosterone-induced increase in gK^+ is significantly less during inhibition of the Na^+-K^+ pump. This suggests that aldosterone can increase gK^+ through dual mechanisms: (i) by stimulation of Na^+/H^+ exchange and (ii) by increasing the activity of the Na^+-K^+ ATPase. The effect of aldosterone on Na^+/H^+ exchange is likely to be a pure mineralocorticoid effect, since in the isolated double-perfused kidney of frogs, the aldosterone-induced increase in luminal acidification of early distal tubule (an indication of increased activity of Na^+/H^+ exchange), was inhibited by spironolactone [46].

Cell pH Is a Signal to Regulate K^+ Conductance

The fused giant cell of the early distal tubule of frog kidney was the first preparation in which stimulation of sodium-hydrogen exchange by aldosterone was demonstrated. These studies showed that one hour after application of aldosterone the intracellular pH significantly increased [34]. Recently it has been observed that aldosterone stimulates Na^+/H^+ exchange and increases intracellular pH in fused

MDCK cells both in the presence and absence of the CO_2/HCO_3^- buffer system [30].

In the present study, cells were incubated for 20–28 hr in aldosterone-containing solution. The magnitude of the intracellular pH increase was less than for acute application of aldosterone [34]. The rise in cell pH can increase the potassium conductance of the apical cell membrane of the same nephron segment [26, 32], due to the activity of pH-sensitive potassium channels [21, 29]. However, increases in single-channel current cannot explain the rise in gK^+ observed in the present study, since during whole-cell voltage-clamping intracellular pH was maintained at 7.3 (due to the presence of 10 mM HEPES in the pipette solution). Our experiments suggest that changes in intracellular pH also act as a signal for cells to regulate membrane channel density. The experiment in which cells were incubated in the absence of aldosterone at pH 8.0 support this hypothesis; gK^+ was almost doubled. A direct activation of pH-sensitive K^+ channels cannot explain this result. Therefore, new channels must have been inserted into the cell membrane. Thus, the elevation of cell pH induced by aldosterone first directly raises open probability of pH-sensitive K^+ channels and secondly, increases the numbers of K^+ channels. Because the amplitude of the aldosterone-induced alkalosis is very large at the beginning of hormone action, the direct effect of pH on K^+ current is an important component in the enlargement of K^+ conductance in the early response to aldosterone. Although incubating cells in high pH solution elevates intracellular pH more than aldosterone treatment, the increase in gK^+ caused by aldosterone is greater. This suggests that aldosterone must have a second effect in addition to stimulating Na^+/H^+ exchange, most likely by activating the Na^+-K^+ pump.

The aldosterone-induced increase in gK^+ can be removed, if cell pH is subsequently reduced. In contrast, if pH is maintained after removal of aldosterone, gK^+ was still significantly higher than that in cells under control conditions even after 8 hours. This result suggests that renal cells have some "memory" after aldosterone treatment and that this memory results from factors such as the changes in intracellular pH and the activity of the Na^+-K^+ ATPase [24].

Aldosterone Stimulates the Na^+-K^+ ATPase

Previous studies report that aldosterone induces an increase of Na^+-K^+ ATPase activity in the distal portions of the nephron [4, 8, 17, 23, 25]. As discussed previously, our observations suggest that a part of the aldosterone-induced increase of gK^+ is due to a stimulation of Na^+-K^+ ATPase activity. The mechanisms of the link between pump activity and K^+ permeability are still not known [38]. Stimulation of the pump may cause falls in both intracellular Na^+ and ATP concentrations, both of which may have effects on the activity of K^+ channels. However, whether long term changes in these factors could cause changes in effective channel number has not been investigated.

Character of the K^+ Channels and Their Role in the Response to Aldosterone

The early distal tubule cells of frog kidney are polarized cells (Fig. 14). However, when renal cells are isolated, it is not possible to determine which membrane of the cells is being patched and to localize channels to a specific membrane. Therefore, the currents of the K^+ channels observed in the present study are most likely derived from both the apical and basolateral cell membranes. The type I channel (with inward conductance of 24 pS) did not carry any detectable outward currents, and the incidence of the channel was not changed after hormone treatment. Thus, this channel cannot contribute to the aldosterone-induced increase in gK^+. The most frequently observed channels under control conditions were the type III K^+ channels (with inward conductance of 59 pS and outward conductance of 34 pS). These channels have some similarities with the K^+ channel described at the basolateral membrane of rabbit proximal convoluted tubule [37]. The K^+ channel of rabbit kidney has an inward conductance of 54 pS and displays similar voltage-dependent opening properties. In the present study, both type I and type III channels were often observed in the same patch. Whether both these K^+ channels are located at the basolateral membrane needs further investigation.

Type II channels (with inward conductance of 45 pS and outward conductance of 21 pS) were found to be inward rectifying, and the ratio between the inward and outward conductance was consistent with the macroscopic gK^+. The insensitivity of type II channels to calcium is congruous with the macroscopic measurements made under calcium-free conditions. Inward-rectifying K^+ channels have been observed in a number of cell types, including rat ventricular myocytes [22], cultured pancreatic islet cells [6], and renal cells [9, 37].

Barium is a well-known blocker of K^+ channels [7, 12, 20, 32, 42]. The sensitivity of the 45-pS K^+ channel to barium is in keeping with the macroscopic measurements, in which all the aldosterone-induced K^+ current was blocked by 2 mM barium.

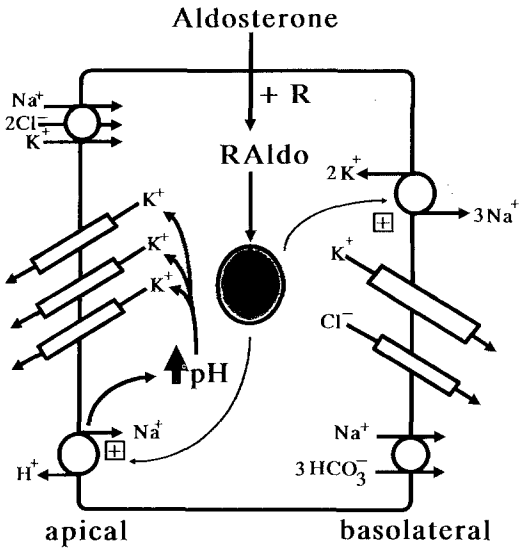


Fig. 15. Scheme for the action of aldosterone on gK^+ in EDC. Aldosterone raises intracellular pH by its action on Na^+/H^+ and the Na^+-K^+ ATPase. The rise in pH increases both single-channel current (in the early phase of its action) and triggers the effective insertion of K^+ channels into the apical membrane. Net K^+ transport switches to the secretory direction

The 45-pS K^+ channel is sensitive to intracellular pH. Increasing the pH of the solution facing the cytoplasmic side of inside-out patches did not change the voltage dependence of channel opening, but shifted the curve to the left. Acidification of the cytoplasmic side of the membrane in excised inside-out patches reduces voltage-dependent open time of Ca^{2+}-activated K^+ channels of pancreatic B-cells and alkalosis has the opposite effect [3]. This sensitivity of channel activity to cell pH may result from competition between Ca^{2+} and H^+ for binding sites. Since the 45-pS channel reported here, is not calcium activated, an independent binding site for H^+ must exist within the channel pore. The inhibition of channel activity by H^+ was voltage dependent. In inside-out patches, altering bath pH at positive potentials did not change single-channel open probability. Whereas, changes in pH at negative potentials were highly effective in changing open probability.

Under control conditions we seldomly observed type II K^+ channels, but after aldosterone treatment they became the most frequently observed channel type. Although we often observed type I and type III channels together in the same patch, we never observed type II channels together with either type I or type III. Under normal conditions, the early distal tubule of amphibian kidney does not secrete K^+ ions. Net secretion of K^+ only occurs during times of increased aldosterone levels (such as accompanies high K^+ adaptation) [28]. Given these observations, it is likely that the normal location of the type II channel is in the apical membrane of early distal cells.

Conclusion

Figure 15 summarizes our view of the cellular action of aldosterone. Aldosterone stimulates both Na^+/H^+ exchange and the Na^+-K^+ ATPase. The activation of the Na^+/H^+ exchanger induced by aldosterone causes an increased sodium influx from lumen to cell. The activity of the Na^+-K^+ ATPase will further increase, to cope with the higher rate of sodium influx, and this results in an increase in the rate of net sodium reabsorption. Increased activity of the Na^+/H^+ exchanger raises intracellular pH and favors exit of bicarbonate from the cell, thus leading to an increased rate of rheogenic Na^+/HCO_3^- cotransport [43, 44]. Intracellular alkalosis in the early phase of aldosterone action can also directly increase potassium conductance [21, 29]. Both raised cell pH and factors associated with the elevated activity of the Na^+/K^+ pump can act as signals to recruit potassium channels into the apical membrane. Thus, the apical membrane potassium conductance is further increased and potassium net flux is changed to the secretory direction.

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