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Effects of vasopressin on single Cl^- channels in the apical membrane of distal nephron cells (A6)

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We have investigated how two types of Cl^- channels found in sodium transporting epithelium are regulated by arginine vasopressin (AVP). A6 cells cultured on permeable supports for 10 to 14 days have two types of Cl^- channels in the apical membrane that have single channel conductances of 3 and 8 pS. In cells without AVP pretreatment, the 3 pS Cl^- channel was more frequently observed than the 8 pS Cl^- channel. AVP increased the open probability (P_o) and single channel conductance of the 3 pS Cl^- channel without significantly changing the P_o or conductance of the 8 pS Cl^- channel. On the other hand, AVP did not affect the number of the 3 pS Cl^- channel, but increased the number of 8 pS Cl^- channels. These observations suggest that AVP has two different pathways to increase apical membrane chloride conductance in distal nephron A6 cells; i.e., (1) increases the P_o and single channel conductance of 3 pS Cl^- channels and (2) increases the number of 8 pS Cl^- channels.

Introduction

It is well known that the distal nephron is one of the target organs (and tissues) of arginine vasopressin (AVP). Specially, the antidiuretic hormonal effect of AVP on distal nephron cells increases the water permeability of the apical membrane of distal nephron cells and increases the water absorption. In addition to increased water permeability, AVP has been shown to increase sodium transport in the distal nephron cells [1,2].

A6 cells, now commonly used as a model of distal nephron cells, respond to various hormones and intracellular second messengers [1–10]. Yanase and Handler [9] and Kleyman and his colleagues [11] have reported that AVP, adenosine cyclic monophosphate (cAMP) as a second messenger of AVP, or forskolin increases transepithelial chloride transport in A6 cells in addition to sodium transport. They have also suggested that the AVP (or cAMP)-activated transepithelial chloride transport occurs through transcellular pathway [9]. More recently, using the patch clamp technique, we have shown that the apical membrane of confluent A6 cells has at least two types of Cl^- channels (single channel conductances of 3 and 8 pS) and that cAMP increases the open probability (P_o) of a 3 pS Cl^- channel [3]. However, the role of the two different types of Cl^- channels in AVP-activated chloride transport is still unclear. In the present study, we investigated how these two types of Cl^- channels are regulated by AVP.

Materials and Methods

The methods and materials were similar to those of our previous study [5]. Briefly, they were as follows.

Cell line and culture methods

A6 cells were purchased from American Type Culture Collection (Rockville, MD, USA) in the 68th passage. All experiments were carried out between the 71st–83rd passages.

Cells were maintained in plastic tissue culture flasks (Corning, New York, NY, USA) at 27°C in a humidified incubator containing 4% CO_2 in air. The culture medium was a mixture of Coon's medium F-12 (three parts) and Leibovitz's medium L-15 (seven parts) modified for amphibian cells with 95 mM NaCl, 25 mM $NaHCO_3$ and pH 7.4. In addition to these components, 1 μM aldosterone (Sigma, St. Louis, MO, USA), 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1.0% streptomycin and 0.6% penicillin (Irvine Scientific, Sant Ana, CA, USA) were present. When cells

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were used for the patch clamp experiment, cells from the flasks were subcultured on permeable supports (Nunc Tissue Culture Inserts, Nunc, Roskilde, Denmark). The patch pipette was applied from the luminal (apical) side and we made a gigaohm seal (> 200 gigaohm).

Patch recording

Standard patch clamp techniques were used [5,12,13]. Patch pipettes were made from LG 16 glass (Dagan Corporation, Minneapolis, MN, USA) and fired-polished to produce tip diameters of approx. 0.5 μm. Single channel currents from cell-attached and excised inside-out patches were obtained at 22–23°C with an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Currents were recorded on a digital video recorder (HF860D, Sony, Tokyo, Japan) with pulse code modulation (1-DR-390, Neuro Data Instruments, New York, NY, USA), and then digitized and analyzed by a Dell computer (325D) with a continuous data acquisition program.

The pipette and patch membrane (> 200 gigaohm) were tightly sealed. A 200 Hz low-pass filter was used. The collection of data for cells treated with AVP (Sigma, St. Louis, MO, USA) was commenced about 10 to 20 min after 50 mU/ml AVP was added from the basolateral side. The average length of recording was about 20 to 30 min.

Determination of the channel number per patch membrane

The number of the channels per patch membrane was determined according to methods we [5] and Hurst and Hunter [14] have previously described. The likelihood method of Hurst and Hunter [14] was applied to an observed amplitude histogram, using a binomial distribution generated from the P_o of individual channels and the total number of channels. From this, we estimated the number of functional channels. Further, if the current levels of all the channels being simultaneously open and closed can be observed, we can determine the real number of functional channels per patch membrane. In regard to the 8 pS Cl^- channel, the P_o was approx. 0.5. This P_o value enables us to make a reliable estimate of the number of 8 pS Cl^- channels per patch membrane (see Discussion). In contrast, the P_o of the 3 pS Cl^- channel was very small in cell-attached patches. However, the P_o of the 3 pS Cl^- channel is regulated by the cytosolic Ca^{2+} concentration [3,4]. By increasing the cytosolic Ca^{2+} concentration (e.g., 1 mM) in excised inside-out patches, we can activate all the 3 pS Cl^- channels in the patch membrane. In the same excised inside-out patches by decreasing the cytosolic Ca^{2+} concentration (e.g., 10 nM), the 3 pS Cl^- channel then had very little activity. Using this strategy of two current levels, with all the 3 pS Cl^- channels either being simultaneously open or closed, we could count the real number of the channels in patch membrane. This enabled us to determine the real number of the 3 pS Cl^- channel per patch (see Discussion).

Open probability (P_o) of the channel

The P_o is calculated by the following equation:

$$P_o = \frac{1}{N}\sum_{i=1}^{N} i \cdot P_i \qquad (1)$$

where N is the number of channels; P_o, the open probability of an individual channel; i, the number of channels simultaneously open; and P_i, the probability of only i channels being simultaneously open. We calculated the P_o, using the number of the channels per patch that was determined by the method described above.

Solutions

The standard bathing and pipette solutions contained 120 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes at a pH of 7.4. The solution of the patch pipette contained 50 μM amiloride (Sigma, St. Louis, MO, USA) to block the amiloride-blockable sodium channels that were frequently observed in the apical membrane of the cell.

Results

To study the effect of arginine vasopressin (AVP) on the Cl^- conductance of the apical membrane of A6 cells, we observed the single Cl^- channel activity before and after AVP (50 mU/ml) was added from the basolateral side while maintaining a cell-attached patch containing Cl^- channel activity.

As previously described [3], the apical membrane of A6 cells cultured on permeable supports has at least two types of Cl^- channels with single channel conductances of 3 and 8 pS. Fig. 1 shows representative traces demonstrating that AVP increased the open probability (P_o) of the 3 pS Cl^- channel. Furthermore, the inward current after the addition of AVP was larger than that in the control (Fig. 1). These observations suggest that AVP has two effects on the 3 pS Cl^- channel: i.e., AVP increases the P_o of the channel and the single channel conductance for the inward current.

Fig. 2 presents the current–voltage (I–V) relationship and the average P_o of the 3 pS Cl^- channel. Fig. 2A indicates that the I–V relationship of the channel was strongly outwardly rectified in control cells (i.e., in the absence of AVP). In cells treated with AVP there was less rectification, due largely to an increase in the inward current (Fig. 2A). AVP did not change the single channel slope conductance between voltage 80

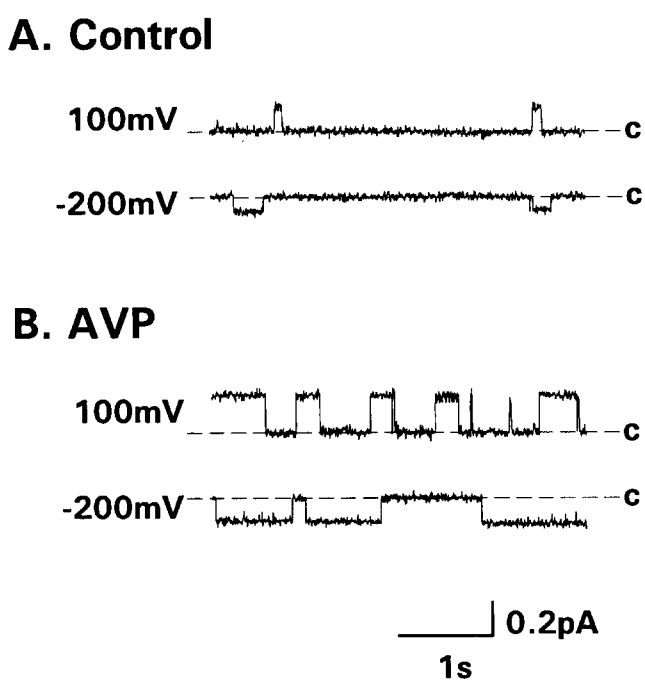

Fig. 1. Single 3 pS Cl^- channel activity from a cell-attached patch before (A, Control) and after (B, AVP) adding arginine vasopressin (AVP) to the basolateral side. Voltage values next to individual records shown in A and B are displacement of intracellular potential of patch membrane from resting membrane potential; e.g., 100 mV means that the patch membrane was 100 mV depolarized from the resting membrane potential. Closed level of single channel within patch is marked with horizontal dash line and 'c' at each trace. Upward deflection indicates outward current across the patch membrane. The mean open and closed times were, respectively, 25 and 3290 ms in control at voltage 100 mV in cell-attached patches. The mean open and closed times after AVP treatment were, respectively, 264 and 219 ms at voltage 100 mV in cell-attached patches.

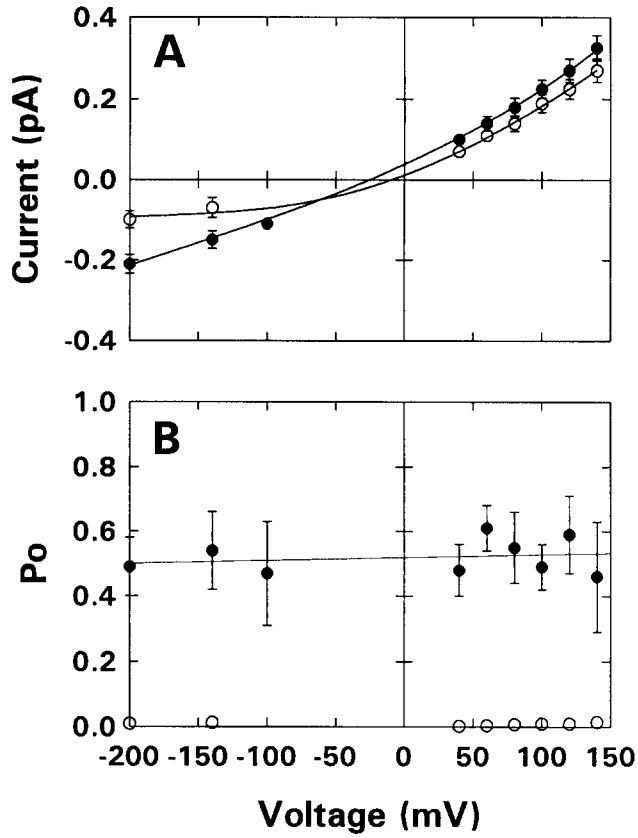

Fig. 2. Current–voltage relationship and the voltage-dependency of the open probability of 3 pS Cl^- channel from cell-attached patches in the presence and absence of AVP in the basolateral solution. (A) The current–voltage relationship in the presence (•) and absence (○) of AVP in cell-attached patches. (B) The open probability (P_o) in the presence (•) and absence (○) of AVP in cell-attached patches. The P_o shown in Fig. 2B was calculated using only the patches showing channel activity; i.e., the patches showing no channel activity were not used for calculation of the P_o. The data are shown as mean values ± S.E. ($n = 3$ (three individual cell-attached patches) for each voltage). Where error bars are not visible they are smaller than the symbol.

mV and 140 mV (outward current): the single channel slope conductance was 2.5 pS in the both cases of the presence and absence of AVP. On the other hand, AVP increased the single channel slope conductance between voltage −200 mV and −140 mV (inward current): it was 0.5 pS in the absence of AVP (control)

TABLE I

The effect of arginine vasopressin (AVP) on the number of the 3 pS and 8 pS Cl^- channels per patch membrane

Channel	Number of channels per patch membrane [a]		Significance
	control ($n = 144$) [b]	AVP ($n = 94$) [b]	
3 pS Cl^-	1.90 ± 0.09	1.85 ± 0.11	n.s.
8 pS Cl^-	0.07 ± 0.03	0.64 ± 0.08	$p < 0.001$

[a] The data are shown as means ± S.E.

[b] n is the total number of all individual patches including the patches which showed no channel activity in cell-attached or excised inside-out configuration. The number of channels was counted from single channel recordings during a period of 20–30 min according to the method described in Materials and Methods. The data for cells treated with AVP were obtained about 10 to 20 min after 50 mU/ml AVP was added. To test for the effect of AVP on the number of the 3 pS and 8 pS Cl^- channels, a Student's t-test was used. Probability (p) < 0.05 was considered significant.

TABLE II

The distribution of the number of patches containing 8 pS Cl^- channels per patch membrane in the presence and absence of AVP

Number of 8 pS Cl^- channels per patch [b]	Number of patches containing 8 pS Cl^- channels [a]	
	control	AVP
0	136	49
1	6	30
2	2	15

[a] The data were obtained from cell-attached patches.

[b] The numbers of channels were counted from single channel recordings during a period of 20–30 min. The data for cells treated with AVP were obtained about 10 to 20 min after 50 mU/ml AVP was added.

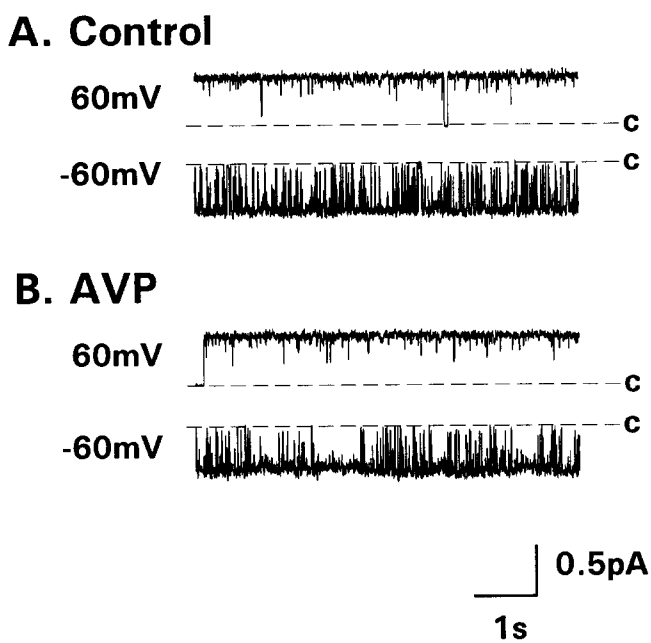

Fig. 3. Single 8 pS Cl^- channel activity from a cell-attached patch before (A, Control) and after (B, AVP) adding AVP to the basolateral solution. The mean open and closed times were, respectively, 552 and 240 ms in control at voltage 60 mV. The mean open and closed times were, respectively, 624 and 211 ms after AVP treatment at voltage 60 mV.

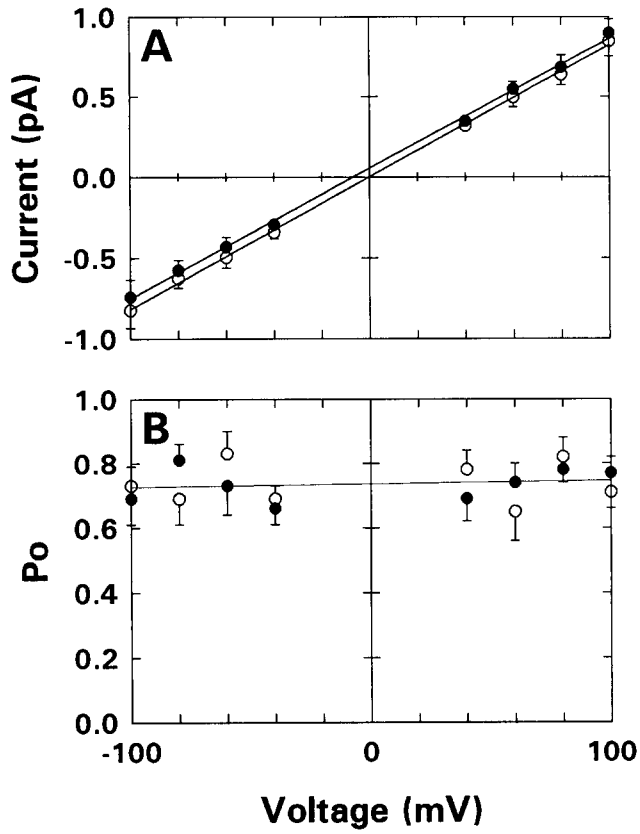

Fig. 4. Current–voltage relationship and the voltage-dependency of the open probability of 8 pS Cl^- channel from cell-attached patches in the presence and absence of AVP in the basolateral solution. (A) The current–voltage relationship in the presence (●) and absence (○) of AVP in cell-attached patches. (B) The open probability (P_o) in the presence (●) and absence (○) of AVP in cell-attached patches. The P_o shown in Fig. 4B was calculated using only the patches showing channel activity; i.e., the patch showing no channel activity was not used for calculation of the P_o. The data are shown as mean values ± S.E. ($n = 3$ (three individual cell-attached patches) for each voltage). Where error bars are not visible they are smaller than the symbol.

and 1 pS in the presence of AVP. The P_o was less than 0.02 in the absence of AVP (control) and about 0.05 in the presence of AVP at all potentials (Fig. 2B).

AVP did not affect the 8 pS Cl^- channel activity or the magnitude of the single channel current (Fig. 3). Fig. 4 shows the I–V relationship and P_o for the channel in the presence and absence of AVP.

Together these findings indicate that AVP increases the P_o and single channel conductance of 3 pS Cl^- channels without any significant effect on 8 pS Cl^- channels. These findings do not, however, show whether AVP has any effect on the number (density) of the channels per unit membrane area. Since macroscopic ion conductance is determined by the number of functional channels, single channel conductance and P_o, we also studied the effect of AVP on the number of functional channels. As shown in Table I, the addition of AVP did not significantly change the number of 3 pS Cl^- channels per patch membrane, but it increased the number of 8 pS Cl^- channels about 9-fold. Table II shows the distribution of the observed number of patch experiments against the number of functional 8 pS Cl^- channels. AVP decreased the number of empty patches and also increased the number of the patches containing functional channels. Table III indicates that AVP did not change the distribution of the observed number of patch experiments against the number of functional 3 pS Cl^- channels.

TABLE III

The distribution of the number of patches containing 3 pS Cl^- channels per patch membrane in the presence and absence of AVP

Number of 3 pS Cl^- channels per patch [b]	Number of patches containing 3 pS Cl^- channels [a]	
	control	AVP
0	16	15
1	36	15
2	50	38
3	30	21
4	12	5

[a] The data were obtained from cell-attached patches.

[b] The numbers of channels were counted from single channel recordings during a period of 20–30 min. The data for cells treated with AVP were obtained about 10 to 20 min after 50 mU/ml AVP was added.

We also conducted experiments in A6 cells that had not treated with high aldosterone and 10% fetal bovine serum ($n = 6$). Although we detected Cl^- channels similar to those seen in cells treated with aldosterone, we did not observe consistent or stable responses to AVP (data not shown).

These observations suggest that AVP increases the macroscopic Cl^- conductance of the apical membrane via two different pathways; AVP increases the P_o and single channel conductance of the 3 pS Cl^- channel and the number (density) of the 8 pS Cl^- channel.

Discussion

In the present study, we report two types of chloride channels (3 pS and 8 pS Cl^- channels) in the apical membrane of A6 cell and their regulation by AVP. Another type of Cl^- channel with a single channel conductance of 400 pS has been reported in these cells [15]. We also observed the same type of the Cl^- channel, however we could observe this channel activity only in subconfluent cells within a couple of days after seeding cells (data not shown). After the cells achieved confluence, we could find only the two types of the Cl^- channels shown in the present and our previous study [3]. The 400 pS Cl^- channel may not contribute to the physiological chloride conductance of the apical membrane. We studied the effect of AVP on two types of the chloride channels (3 pS and 8 pS) to clarify the regulatory mechanism of the AVP-induced increase in macroscopic chloride conductance of the apical membrane (transepithelial chloride transport).

We should argue whether AVP really increases the number of the 8 pS Cl^- channel. Table II shows the distribution of the number of the 8 pS Cl^- channels observed per patches. In control cells, 136 out of 144 patches showed no channel activity. We observed the channel activity at least for 20 min at each patch experiment, even when we could not observe any channel activity (or current transition). As shown in our previous report [3], the 8 pS Cl^- channel has two closed states; i.e., the mean closed times are approx. 10 and 1000 ms. Even assuming that the channel only has the longer mean closed time, the probability of the channel staying closed for 1 min is $8.76 \cdot 10^{-27}$. This means that the probability of missing any opening events is $8.76 \cdot 10^{-27}$, which is very unlikely. This indicates that we did not fail to observe opening events of the 8 pS Cl^- channel even in 1-min recording period if the patch membrane would the functional 8 pS Cl^- channel. Our observation of 136 patches without detectable 8 pS Cl^- channel activity, therefore, is likely accurate.

Similar arguments can be applied to other patches; 6 and 2 out of 144 patches in the control cells contained one and two functional channels, respectively. Even though we did not observe more than one channel open in 6 patches (Table II), it is possible that there are two functional channels and that it is only by chance that we failed to observe two simultaneous open channels. The mean open time was about 0.5 s. So, even assuming that the channel only has the longer mean closed time (i.e., 1 s), the expected number of open events for 1-min recording period is 40 [60/(0.5 + 1) = 40]. For each event in which we observe a single channel open, the probability, P, that the open channel will close before the second channel opens is expressed as the following equation [5].

$$P = \frac{\frac{1}{T_o}}{\frac{1}{T_o} + \frac{(N-1)}{T_c}} \qquad (2)$$

where T_o and T_c are the mean open and closed times, respectively, and N is the number of functional channels. In this example, N is 2. So, P is 0.667 (2/3). Therefore, the probability that each of those 40 events (for 1-min recording time) would end with the first channel closing before the second channel opens is $9 \cdot 10^{-8}$ or $(2/3)^{40}$. This value is small enough for us to conclude that 6 patches only had one functional channel.

A similar argument can be used for two patches containing two function channels shown in Table II. This argument indicates that the number of 8 pS Cl^- channels per patch in control shown in Table I is not underestimated. We can, therefore, conclude that AVP increases the number of functional 8 pS Cl^- channels.

We should also argue whether AVP really had no influence upon the number of the 3 pS Cl^- channel. Table III shows the distribution of the number of the 3 pS Cl^- channels observed per patches. In control cells, 16 out of 144 patches showed no channel activity. We observed the channel activity at least for 20 min at each patch experiment, even when we could not observe any channel activity (or current transition). The mean closed time is 3290 ms at a potential 100 mV more positive than the resting potential in cell-attached patches. The probability of the channel staying closed for 1 min is $1.20 \cdot 10^{-8}$. This means that the probability of missing any opening events is $1.20 \cdot 10^{-8}$, which is very unlikely. AVP decreased the mean closed time of the 3 pS Cl^- channel, indicating that the probability of the channel staying closed for 1 min is less than $1.20 \cdot 10^{-8}$. This means that the probability of missing any opening events is less than $1.20 \cdot 10^{-8}$, which is also very unlikely after adding AVP. Our observation of 16 (control) and 15 (AVP) patches without detectable 3 pS Cl^- channel activity, therefore, is likely accurate.

Similar arguments can be applied to other patches; e.g., 36 out of 144 patches in the control cells contained one functional 3 pS Cl^- channels. Even though we did not observe more than one channel open in 36 patches (Table III), it is possible that there are two functional channels and that it is only by chance that we failed to observe two simultaneous open channels. The mean open time was about 25 ms at a potential 100 mV more positive than the resting potential in cell-attached patches. The expected number of open events for 20-min recording period is 362.5 [$20 \times 60/(0.02 + 3.29) = 362.5$]. For each event in which we observe a single channel open, the probability, P, that the open channel will close before the second channel opens is expressed by Eqn. 2. In this case, N is 2. So, P is 0.9925. Therefore, the probability that each of those 362.5 events (for 20-min recording time) would end with the first channel closing before the second channel opens is 0.065 or $0.993^{362.5}$, indicating that we might fail to observe two simultaneous open channels with a probability of 0.065. However, a rise in the cytosolic Ca^{2+} concentration in excised inside-out patches causes an increase in the P_o of the 3 pS Cl^- channel [3,4] that can reduce the possibility that we fail to observe two simultaneous open channels. For example, the mean open and closed time were, respectively, 500 and 18 ms at a holding potential of 100 mV with the cytosolic Ca^{2+} concentration of 1 mM in excised inside-out patches. These values give us the probability of $6.81 \cdot 10^{-29}$ to fail to observe the simultaneous open of two channels even in 10-s record. This argument can be applied to the determination of the number of AVP-activated 3 pS Cl^- channels. We therefore conclude that 36 and 15 patches only had one functional channel in control and AVP-treated cells, respectively.

A similar argument can be used for other patches containing more than one function channel shown in Table III. In conclusion, the number of 3 pS Cl^- channels determined by using such a high cytosolic Ca^{2+} concentration in excised inside-out patches did not differ from that shown in Table I; namely, the number of 3 pS Cl^- channels per patch in control shown in Table I is not underestimated. We can, therefore, conclude that AVP does not affect the number of functional 3 pS Cl^- channels.

AVP reduced the rectification of the 3 pS Cl^- channel as a result of the increased inward current (Fig. 2A). This contrasts with our previous study [3] indicates that the intracellular second messenger of AVP, cAMP, did not change the rectification of the 3 pS Cl^- channel. These observations suggest that AVP could decrease the outward rectification of the 3 pS Cl^- channel through GTP-binding protein-mediated pathway but not through cAMP-dependent protein kinase-mediated pathway.

The observations shown in the present report indicate that AVP has two effects on the Cl^- channels: AVP increases the P_o of 3 pS Cl^- channels, but does not change the number of the channels, and AVP increases the number of the 8 pS Cl^- channels, but does not change the P_o of the channel. These observations of the effect of AVP on the 3 pS and 8 pS Cl^- channels suggest that AVP increases macroscopic Cl^- conductance of the apical membrane via regulation of both types of Cl^- channels.

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