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Short communication

Blocking action of cytochalasin D on protein kinase A stimulation of a stretch-activated cation channel in renal epithelial A6 cells

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Abstract

We have shown that the apical membrane of renal epithelial A6 cells has a 29-pS stretch-activated nonselective cation (NSC) channel, which is activated by cytosolic cyclic AMP (cAMP) (J Gen Physiol 1997;110:327–36). In general, downstream signalings of cAMP are mediated through a cAMP-activated protein kinase (protein kinase A, PKA)-dependent pathway. Therefore, to study if the channel is activated by a PKA-dependent pathway, we applied a PKA catalytic subunit directly to the channel from the cytosolic surface in cytosol-free excised inside-out patches, using the single channel recording (patch clamp) technique. Application of PKA catalytic subunit with 2 mM ATP increased the open probability (P_o) of the channel from 0.11 ± 0.04 to 0.58 ± 0.10 (mean ± SD, N = 11, $P < 0.001$). The channel has a gating kinetics "$C_L \leftrightarrow C_S \leftrightarrow O$," where C_L, C_S, and O are the long closed state, the short closed state, and the open state, respectively. PKA influenced the communication of the channel between C_L and C_S without affecting the communication between C_S and O, leading the channel to only stay in C_S and O. The PKA-induced increase in P_o was attributable to the interruption of communication between C_L and C_S or to the reduction of time the channel stays in C_L. Pretreatment with cytochalasin D (Cyt-D), an inhibitor of the polymerization of actin filaments, blocked the stimulatory effect of PKA on the channel. These observations suggest that phosphorylation of polymerized actin filaments regulates the gating kinetics of a stretch-activated channel in renal epithelium.

Keywords: Protein kinase A; Single channel current; Gating kinetics; Nonselective cation channel; Patch clamp; Actin filament

1. Introduction

Renal epithelial A6 cells have been used as a model cell line for studies on ion transport in the distal nephron epithelium [1–6]. A6 cells have a 29-pS stretch-activated NSC channel, which is also activated by an inhibitor of phosphodiesterase, IBMX. Treatment with IBMX increases the cytosolic cAMP concentration in A6 cells, suggesting that a cAMP-dependent pathway, such as PKA, is involved in the stimulation of the NSC channel [7]. However, it is still unknown if PKA is really involved in the stimulatory action of IBMX on the NSC channel. Our previous study [7] reported that Cyt-D, an inhibitor of the polymerization of actin filaments, blocks the stimulatory action of IBMX on the NSC channel. Whether Cyt-D blocks the stimulatory action of PKA on the NSC channel, however, remains to be determined. In the present study, we found that PKA activated the NSC channel and that Cyt-D blocked the action of PKA. In addition, we demonstrated the effect of PKA on the gating kinetics of the channel, and the blocking action of Cyt-D on this effect.

2. Materials and methods

2.1. Solutions

The pipette solution contained 120 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). The bathing (cytosolic) solution contained 120 mM KCl, 100 nM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4).

2.2. Cell culture

A6 cells in the 68th plating were purchased from the American Type Culture Collection. All experiments were

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Abbreviations: NSC, nonselective cation; cAMP, cyclic AMP; PKA, cAMP-activated protein kinase (protein kinase A); IBMX, 3-isobutyl-1-methylxanthine; and Cyt-D, cytochalasin D.

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performed on the 71st-83rd platings of the cells, which were subcultured on a permeable filter support (Nunc Tissue Culture Inserts) [8–10].

2.3. Patch recording

As previously described [11–14], single-channel currents were obtained at 22–23°, applying standard patch-clamp techniques. A 2000-Hz low-pass filter was used to analyze the channel kinetics. The patch pipette was applied from the luminal side, and a gigaohm seal (>100 gigaohms) was made on the apical membrane of the cells.

2.4. P_o of a single channel

Channel activity is expressed as P_o

$$P_0 = \frac{T_0}{T_t} \quad (1)$$

where T_t is the total recording time and T_o is the total time the channel is open during the total recording time period in a patch. To study the P_o, patches that contained only one channel in an excised inside-out patch were used.

2.5. Application of PKA and Cyt-D

When the NSC channel was treated with PKA, PKA (10 μg/mL) was applied to the cytosolic surface of excised inside-out patches in the presence of 2 mM ATP. To study the effect of Cyt-D on PKA action, A6 cells were pretreated with Cyt-D (5 μM, 10 min) before the formation of excised inside-out patches.

2.6. Chemicals and statistical analyses

All chemicals were purchased from the Sigma Chemical Co., unless otherwise indicated. Results are presented as means ± SD. Statistical significance was tested using Student's *t*-test or ANOVA, as appropriate. A P value of < 0.05 was considered significant.

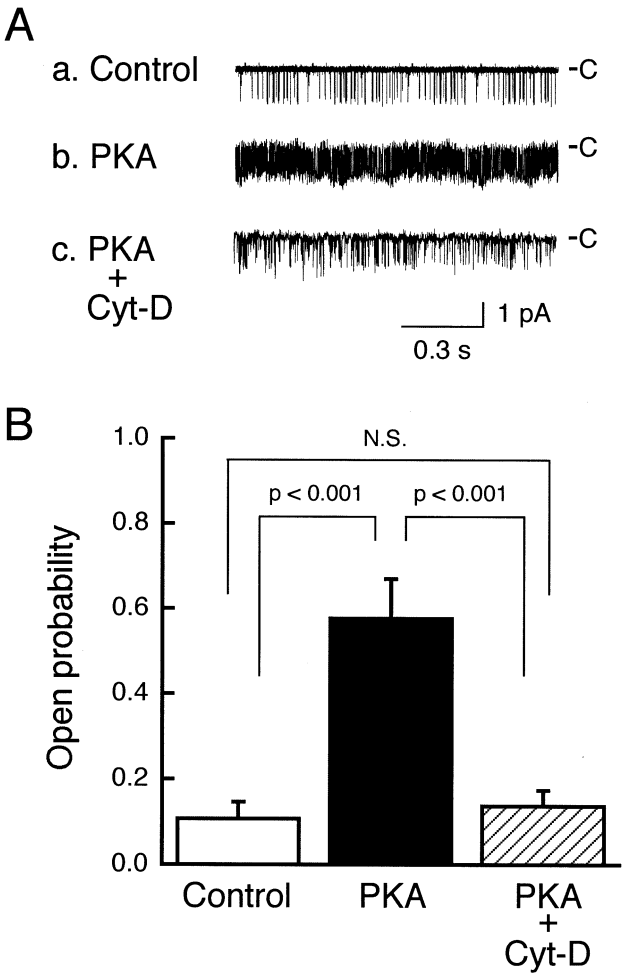

Fig. 1. Effects of PKA catalytic subunit and Cyt-D on the P_o of the NSC channel obtained from excised inside-out patches formed on the apical membrane at a membrane potential of −40 mV in the presence of 2 mM ATP. (A) Representative traces of the single-channel currents before treatment (a. Control), 10 min after application of PKA (10 μg/mL) (b. PKA), and 10 min after PKA application with Cyt-D pretreatment (5 μM, 10 min) (c. PKA + Cyt-D) to the cytosolic surface of the patch membrane. The closed level of the single channel within the patch is marked by a horizontal bar and "C." (B) Statistical result of the effects of the PKA catalytic subunit and Cyt-D on the P_o. The PKA catalytic subunit significantly increased the P_o, and Cyt-D pretreatment significantly decreased the P_o to a level identical to the control; there was no significant difference between the control and PKA plus Cyt-D. Values are means ± SD, N = 11.

3. Results and discussion

Since cAMP activated the NSC channel in A6 cells, we studied the effect of PKA on the activity of the channel (P_o). PKA increased the P_o of the channel (Fig. 1). The activity of stretch-activated channels is known to be related to actin filaments, one type of cytoskeleton [15]. Therefore, to study if actin filaments play a critical role in the PKA activation of the channel, an inhibitor of actin filament polymerization, Cyt-D, was applied to the A6 cells. Cyt-D abolished the stimulatory action of PKA on the P_o (Fig. 1). Figure 2 shows the open and closed time-interval histograms under various conditions, i.e. control (Fig. 2A), PKA treatment (Fig. 2B), and PKA plus Cyt-D treatment (Fig. 2C). These histograms indicate that the NSC channel had one open and two closed states (see Fig. 2, panels A and C) except after PKA treatment where only one open time and one closed time were observed (Fig. 2B). Using the same method as previously reported [7], we obtained the values of the mean open and closed times, fractional numbers of events, and fractional times of open and closed events, as shown in Table 1. The action of PKA was mediated through modification of the frequency of closed events without any significant effects on the open event (Table 1), i.e. PKA increased the frequency and fractional time of the short closed event

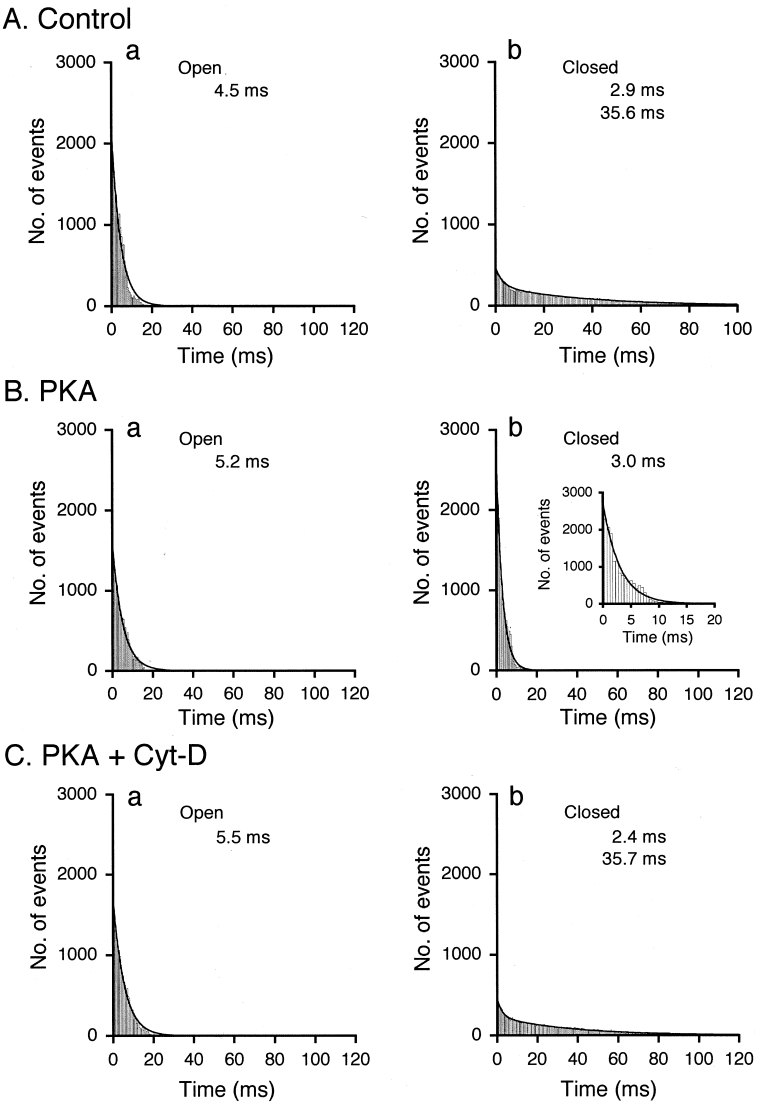

Fig. 2. Effects of the PKA catalytic subunit and Cyt-D on the open and closed time-interval histograms of the NSC channel obtained from excised inside-out patches formed on the apical membrane at a membrane potential of −40 mV in the presence of 2 mM ATP. Each histogram was generated from a 10-min record. (A) Representative open (a) and closed (b) histograms without any treatment. (B) Representative open (a) and closed (b) histograms 10 min after application of PKA (10 μg/mL). (C) Representative open (a) and closed (b) histograms 10 min after PKA application with Cyt-D pretreatment (5 μM, 10 min).

and abolished the long closed event. Cyt-D almost completely abolished the action of PKA (Table 1). Our previous study [7] reported that the channel has a gating kinetics as follows:

$$C_L \underset{k_{-L}}{\overset{k_L}{\rightleftharpoons}} C_S \underset{k_{-S}}{\overset{k_S}{\rightleftharpoons}} O \qquad (2)$$

where C_L is the long closed state; C_S, the short closed state; O, the open state; and k, the leaving rate from each state. Based upon the gating kinetics described above, we determined the gating kinetics using the same method as previously reported [7]. In the control and PKA with Cyt-D-treated experiments, the values of k_L, k_{-L}, k_S and k_{-S} were determined by the exact method described previously [7]. The estimated value of each rate is shown in Table 2. In the case of PKA (unlike the other cases), we observed only one closed time. There are two possibilities to explain this phenomenon: (i) the long closed state really disappeared (in other words, the channel does not have access to the long closed state and has only one closed state), or (ii) the mean time of the channel staying at the long closed state becomes identical to that of the short closed state (in other words, based upon only the mean times the long closed state cannot be distinguished from the short closed state, hence giving the impression that the channel has only one closed state). Even in the latter case and similar to the former case, it is suggested that PKA affects the transition rate of the channel from the short closed state to the long closed state, and that PKA action on this rate is blocked by treatment with Cyt-D. This observation means that PKA-induced phosphorylation

Table 1
The Mean open and closed times, the fractional number of events, and the fractional time of each state of the basal (control) and PKA-activated NSC channels with and without Cyt-D treatment

	Open	Closed	
		Short	Long
Mean times (msec)			
Control	41.1 ± 1.1	3.6 ± 1.3	36.5 ± 3.7
PKA	4.9 ± 2.0	3.5 ± 0.7	N/O
PKA with Cyt-D	4.8 ± 2.0	2.9 ± 1.2	33.5 ± 4.8
Fractional number of events (%)			
Control	50.1 ± 0.6	2.8 ± 0.8	47.1 ± 1.0
PKA	49.9 ± 0.3	50.1 ± 0.8*	N/O
PKA with Cyt-D	50.2 ± 0.7	4.7 ± 1.6**, ***	45.1 ± 1.8**
Fractional time (%)			
Control	10.7 ± 3.9	0.5 ± 0.2	88.8 ± 4.1
PKA	57.7 ± 9.4*	42.3 ± 9.4*	N/O
PKA with Cyt-D	13.6 ± 3.7***	0.8 ± 0.4**, ****	85.6 ± 3.8****

N/O means that the event was not observed. Values are means ± SD, N = 11.
*$P < 0.001$, compared with the control.
**$P < 0.005$, compared with the control.
***$P < 0.01$, compared with PKA.
****$P < 0.05$, compared with the control.

of polymerized actin filaments influences the communication of the channel between the long and short closed states, and that the PKA action requires polymerization of actin filaments. In addition to the effect of Cyt-D on actin filaments we also have to consider that Cyt-D can influence various factors (e.g. CD4 cross-linking [16]), which might regulate the channel activity.

The present study clearly concludes that phosphorylation by PKA influences only the communication between the short and long closed states without having any effect on the open state. In contrast to PKA treatment, we still observed the long closed state with stretch [7]. Despite this, the phenomenon caused by PKA treatment is qualitatively identical to the action of stretch on the channel kinetics [7]. Namely, both the PKA treatment and stretch influence only the communication between the short and long closed states without affecting the open state. In the case of stretch, protein phosphatases would still play an essential role in the dephosphorylation of proteins, resulting in incomplete phosphorylation of proteins by PKA. On the other hand, in the case of PKA treatment, the experiments were performed in cytosolic-free excised inside-out patches, and under this condition the PKA treatment would cause relatively complete phosphorylation of proteins compared with the case of stretch. These different experimental conditions would cause the quantitative differences observed in the cases of PKA and stretch. This suggests that the communication of the channel between the short and long closed states is affected by PKA-induced phosphorylation of polymerized actin filaments. These observations and our previous study [7] indicate that phosphorylation of polymerized actin filaments mimics the action of stretch, suggesting that phosphorylation might produce some stretch-like mechanical phenomenon.

Table 2
Rates leaving from each state in the basal (control) and PKA-activated NSC channels with and without Cyt-D treatment

	Rate (sec^{-1})			
	k_L	k_{-L}	k_S	k_{-S}
Control	28 ± 5	5589 ± 2268	310 ± 98	287 ± 126
PKA	N/E	N/D	301 ± 100	227 ± 83
PKA with Cyt-D	30 ± 4	4390 ± 3066	372 ± 108	226 ± 65

N/E means that the rate could not be estimated. N/D means that the value of k_{-L} is negligibly smaller or much larger than that of k_S. In the latter case, the value of k_L is also much larger than that of k_S. There were no significant differences between the rates of the control NSC channel and the PKA-activated NSC channel with Cyt-D treatment. Neither k_S nor k_{-S} was affected by PKA treatment. Values are means ± SD, $N = 11$.

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