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Flow-Dependent Activation of Maxi K^+ Channels in Apical Membrane of Rabbit Connecting Tubule

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Abstract. The Ca^{2+}-activated maxi K^+ channel was found in the apical membrane of everted rabbit connecting tubule (CNT) with a patch-clamp technique. The mean number of open channels (NP_o) was markedly increased from 0.007 ± 0.004 to 0.189 ± 0.039 ($n = 7$) by stretching the patch membrane in a cell-attached configuration. This activation was suggested to be coupled with the stretch-activation of Ca^{2+}-permeable cation channels, because the maxi K^+ channel was not stretch-activated in both the cell-attached configuration using Ca^{2+}-free pipette and in the inside-out one in the presence of 10 mM EGTA in the cytoplasmic side. The maxi K^+ channel was completely blocked by extracellular 1 μM charybdotoxin (CTX), but was not by cytoplasmic 33 μM arachidonic acid (AA). On the other hand, the low-conductance K^+ channel, which was also found in the same membrane, was completely inhibited by 11 μM AA, but not by 1 μM CTX. The apical K^+ conductance in the CNT was estimated by the deflection of transepithelial voltage (ΔV_t) when luminal K^+ concentration was increased from 5 to 15 mEq. When the tubule was perfused with hydraulic pressure of 0.5 KPa, the ΔV_t was only −0.7 ± 0.4 mV. However, an increase in luminal fluid flow by increasing perfusion pressure to 1.5 KPa markedly enhanced ΔV_t to −9.4 ± 0.9 mV. Luminal application of 1 μM CTX reduced the ΔV_t to −1.3 ± 0.6 mV significantly in 6 tubules, whereas no significant change of ΔV_t was recorded by applying 33 μM AA into the lumen of 5 tubules ($\Delta V_t = -7.2 \pm 0.5$ mV in control *vs.* $\Delta V_t = -6.7 \pm 0.6$ mV in AA). These results suggest that the Ca^{2+}-activated maxi K^+ channel is responsible for flow-dependent K^+ secretion by coupling with the stretch-activated Ca^{2+}-permeable cation channel in the rabbit CNT.

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Key words: Flow-dependent K^+ secretion — Stretch-activation — Charybdotoxin — Arachidonic acid — Luminal membrane

Introduction

In vivo micropuncture and microperfusion studies revealed that renal K^+ excretion is regulated in the distal nephron segments, including distal convoluted tubule (DCT), connecting tubule (CNT) and cortical collecting duct (CCD) (Malnic, Klose & Giebisch, 1966; Good & Wright, 1979; Stanton & Giebisch, 1982). By using patch-clamp technique, both the maxi K^+ channel and the low-conductance K^+ channel were found in the apical membrane of the rabbit (Hunter et al., 1984) and rat CCD (Frindt & Palmer, 1987, 1989). The properties of these two types of K^+ channels were compared to clarify their role in the K^+ secretion along the CCD. Open probability of the maxi K^+ channel demonstrated in cell-attached configuration was too low to play a role in K^+ secretion in the CCD, and their density estimated from the frequency of observation was also too low, whereas both properties of the low-conductance K^+ channel were very high (Wang, Schwab & Giebisch, 1990). In addition, tetraethyl-ammonium (TEA), which markedly reduced the size of single maxi K^+ channel current, made no significant change in whole-cell current recorded from the principal cell (Pácha et al., 1991) which is known to be responsible for the K^+ secretion along the CCD (Stanton et al., 1981; Kaissling & Le Hir, 1982). On the basis of these observations, the low-conductance K^+ channel, which has been cloned as a member of ROMK channel family (Ho et al., 1993; Hebert, 1995), is considered to be a K^+ secretory pathway in the apical membrane of the CCD.

On the other hand, the maxi K^+ channel has been reported to be activated by stretching the patch mem-

brane or exposing it to a hypo-osmotic solution in various renal tubules (Kawahara, Ogawa & Suzuki, 1991; Hirsch et al., 1993) or cultured cell (Taniguchi & Guggino, 1989). Due to the low activity in the unstretched condition, their role was thought to be an emergency pathway for K^+ extrusion from cytoplasm in hypo-osmotic cell volume regulation (Giebisch, 1995). Our recent study in the rabbit CNT perfused in vitro (Taniguchi et al., 1994), however, demonstrated that an enhancement of luminal fluid flow by increasing perfusion pressure in physiological range clearly stimulated apical Ca^{2+} entry into cytoplasm via stretch-activated Ca^{2+}-permeable cation channels. This observation is very important to discuss the role of maxi K^+ channels, because the activity of maxi K^+ channels was not small in the stretched condition as stated above. The coupling of maxi K^+ channel with the stretch-activated cation channel may contribute to the physiological pathway for K^+ secretion and to the formation of apical membrane voltage (V_a) in the distal nephron, as suggested by Taniguchi and Guggino (1989) and Taniguchi et al. (1994).

This hypothesis is consistent with the well-known phenomenon observed by in vivo microperfusion experiments that distal K^+ secretion is correlated with the flow rate of luminal fluid (Kunau, Webb & Borman, 1974; Khuri et al., 1975; Good & Wright, 1979). Furthermore, Okusa, Velázquez and Wright (1991) reported that the positive shift of transepithelial voltage (V_t) in response to a luminal application of amiloride was very small in the absence of luminal Ca^{2+}, and that an addition of Ca^{2+} positively shifted V_t in a flow-dependent manner. This flow-dependent positive shift of V_t was accelerated by a higher concentration of luminal Ca^{2+}. Their observations suggested that the flow-dependent increase in Ca^{2+} entry hyperpolarized the apical membrane of the distal nephron by stimulating the maxi K^+ channel activity. Thus, it is necessary to clarify the role of maxi K^+ channels in the apical membrane of the distal nephron in the presence of physiological luminal fluid flow.

In this study, we investigated the stretch-activation of maxi K^+ channels and the effect of charybdotoxin (CTX) on these channels in the apical membrane of the rabbit CNT where we had already confirmed the existence of stretch-activated channels and the flow-dependent apical Ca^{2+} entry (Taniguchi et al., 1994). We estimated apical K^+ conductance by measuring a deflection of V_t (ΔV_t) upon an abrupt increase in the luminal K^+ concentration. Then, the flow-dependence of ΔV_t and the effect of CTX on it were studied to clarify the nature of flow-dependent activation of maxi K^+ channels and their role in the distal K^+ secretion. Because arachidonic acid (AA) was reported to inhibit the low-conductance K^+ channel activity in the rat CCD, we examined the contribution of two types of K^+ channels to the apical K^+ conductance by comparing the effect of AA on ΔV_t with that of CTX.

Materials and Methods

Preparation of CNT

CNT was isolated from male Japanese white rabbit weighing 2.0–2.5 Kg, according to the criteria described by Imai (1979). In brief, kidney was removed from the animal anesthetized with pentobarbital sodium (35 mg/Kg, i.v.). It was sliced about 1 mm in width, then the slices were placed at 4°C in the modified Collins solution containing (in mM): 14.0 KCl, 44.0 K_2HPO_4, 14.0 KH_2PO_4, 9.0 $NaHCO_3$, and 160.0 sucrose, pH7.4. The CNT was dissected from the slice with fine forceps under a stereomicroscope.

For patch-clamp experiments of the apical membrane, the CNT was everted with a microperfusion apparatus (Narishige, Tokyo, Japan), as described previously (Engbretson, Beyenbach & Stoner, 1988; Taniguchi et al., 1994). The tubule was drawn into an outer perfusion pipette. The end of the tubule was picked up with an inner perfusion pipette, then it was pushed into its own lumen by advancing the inner pipette with applying gentle suction to the outer pipette. The inner pipette was forwarded until the everted tubule was completely exposed from the other end of the tubule. We did not pull out an inserted inner pipette from the everted CNT to avoid serious damage to the tubule. Because the tubule was tightly held with the inner pipette, tubular movement by flow turbulence of bathing solution was minimized. The eversion of the tubule enabled an easy approach of patch pipette on the apical membrane of the CNT.

Patch-Clamp Study

Single-channel current was recorded from apical membrane of everted CNTs at 36°C with a patch-clamp amplifier (EPC-7, HEKA Elektronik, Lambrecht/Pfalz, Germany) (Hamill et al., 1981). For cell-attached and inside-out patch-clamp experiments, patch pipettes (3–5 MΩ) were filled with the solution containing (in mM): 140.0 NaCl, 1.0 $CaCl_2$, 10.0 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), of which pH was adjusted to 7.4 by adding tris(hydroxymethyl)aminomethane (Tris). In cell-attached patch-clamp experiments, the bath was perfused with modified Ringer solution containing (in mM): 110.0 NaCl, 25.0 $NaHCO_3$, 5.0 KCl, 1.0 NaH_2PO_4/Na_2HPO_4, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10.0 Na acetate, 5.6 glucose, 5.0 alanine. This solution was bubbled with 95% O_2/5% CO_2 to adjust pH at 7.4. To stretch the patch membrane, negative pressure (−5 KPa) was applied into patch pipette which was connected to hydraulic manometer.

In inside-out patch-clamp experiments, the bath was perfused with the solution containing (in mM): 140.0 KCl, 10.0 ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA), 10.0 HEPES/Tris, pH7.4. To activate maxi K^+ channels, $CaCl_2$ was added at 9.2, 9.49 or 9.6 mM into the above solution, in which free Ca^{2+} concentration was calculated as 0.5, 0.8 or 1 μM. For recording of low-conductance K^+ channel current, no $CaCl_2$ was added in the bathing solution to inactivate maxi K^+ channels.

In outside-out patch-clamp experiments, patch pipettes were filled with the solution containing (in mM): 140.0 KCl, 10.0 HEPES/Tris, pH7.4. $CaCl_2$ was added at 0.1 mM into this solution for recording of maxi K^+ channel current, whereas 10.0 mM EGTA was added for recording of low-conductance K^+ channel current. In these experiments, bath was perfused with modified Ringer solution.

Single-channel currents were stored with an FM tape recorder (XR30, TEAC, Tokyo, Japan). Recordings of maxi K^+ channel current were filtered with 4-pole Bessel low-pass filter (E-3201A, NF Electronic Instruments, Yokohama, Japan) at cutoff frequency (f_c) of 1 KHz, and recordings of low-conductance K^+ channel current were filtered at f_c of 0.5 KHz. Both of them were digitized at 2 KHz with a TL-1 DMA interface (Axon Instruments, Foster City, CA). We analyzed them with pCLAMP software (Axon Instruments). Mean number of open channel (NP_o) was obtained from 10–30-sec recordings of single-channel currents. Because the number of open channels widely varied in every patch, we normalized NP_o in each condition by control NP_o, and described it as relative NP_o. An outward current from the cytoplasmic side of the patch membrane to the extracellular side was described upwardly and positively, and vice versa for an inward current.

Measurement of V_t

V_t was measured from an isolated rabbit CNT at 36°C with in vitro microperfusion technique according to the method of Burg et al. (1966) as modified by Shimizu et al. (1990*a*). The tubule bathed in modified Ringer solution was hooked up to a set of perfusion pipettes equipped on a microperfusion apparatus (Narishige). Then, the tubular lumen was perfused from an inner perfusion pipette with the same Ringer solution. A calomel half cell was connected to a lumen of the inner perfusion pipette via 1M NaCl agar bridge. We recorded V_t from this calomel electrode by using EPC-7 (HEKA Elektronik Instrument) in current clamp mode. The bathing solution was grounded via another set of 1M NaCl agar bridge and calomel half cell. Perfusion pressure was varied by changing the height of the fluid reservoir connected to the inner perfusion pipette. The exchange of luminal solution was checked by adding FD&C green dye into the luminal solution alternately.

Drugs

CTX was purchased from Peptide Institute (Osaka, Japan) and was dissolved in pure water at 0.1 mM. AA and disodium salt of adenosin 5′-triphosphate (ATP) was purchased from Sigma (St. Louis, MO). We dissolved AA in ethanol at 33 mM, and dissolved Na_2ATP at 100 mM in the solution containing (in mM): 140.0 NaCl, 1.0 $CaCl_2$, 10.0 HEPES/Tris, pH7.4. These drugs were stored at −60°C and were diluted with appropriate solution before experiments.

Statistical Analysis

The data were expressed as mean ± SE (n = number of experiments). Statistical significance was evaluated by either Student's t-test or multiple comparison (ANOVA). The P value less than 0.05 was considered to be significant.

Results

Existence of Maxi K^+ Channels

Figure 1*A* shows single-channel currents with large amplitude which were recorded from an inside-out patch membrane excised from the apical membrane of an everted rabbit CNT. These currents were only recorded outwardly at membrane voltages (V_m) between −60 and +80 mV. Because Na^+ and Ca^{2+} ions existed only in the extracellular side of patch membrane (patch pipette), they could not carry an outward current. Cl^- current must be reversed at 0 mV, because Cl^- concentration was symmetric between both sides of patch membrane. Thus, these outward currents must be carried by K^+ which existed only in cytoplasmic side (bath) in this experimental condition.

We examined the voltage-dependence and the effect of cytoplasmic free Ca^{2+} on NP_o of these channels (Fig. 1*B*). In the presence of 0.8 μM Ca^{2+} in the cytoplasmic

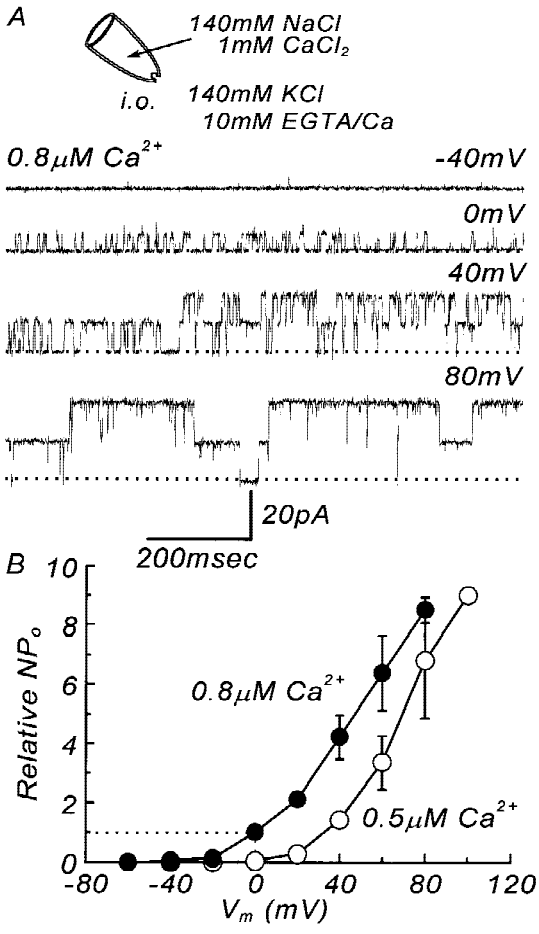

Fig. 1. Ca^{2+}- and voltage-dependence of maxi K^+ channels. (*A*) Single channel currents were recorded from inside-out (i. o.) patch. Patch pipette solution (in mM): 140.0 NaCl, 1.0 $CaCl_2$, 10.0 HEPES/Tris, pH7.4. Bathing solution (in mM): 140.0 KCl, 10.0 EGTA/$CaCl_2$, 10.0 HEPES/Tris, pH7.4. Free Ca^{2+} concentration was shown in the figure. The same solutions were used in all the inside-out experiments otherwise mentioned. (*B*) Relation between relative NP_o and patch membrane voltage in 4 experiments in the presence of 0.5 (open circles) or 0.8 μM Ca^{2+} (closed circles) in the bath. NP_o was normalized as relative NP_o by the NP_o (0.606 ± 0.231, n = 4) obtained at 0 mV in the presence of 0.8 μM Ca^{2+}.

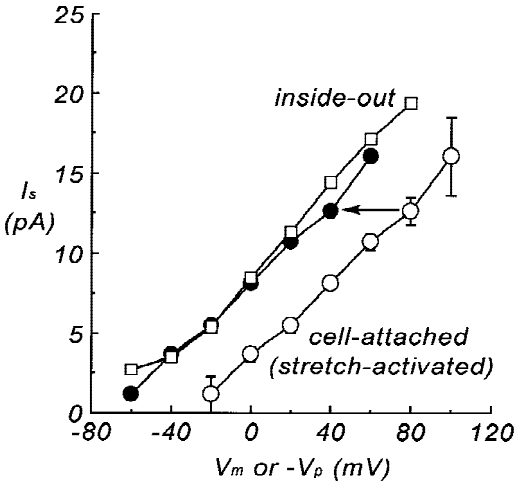

Fig. 2. *IV* relation of single maxi K^+ channel currents recorded from inside-out patch depicted in Fig. 1 (open squares, plotted against V_m, $n = 9$) and from cell-attached patch depicted in Fig. 3 (open circles, plotted against $-V_p$, $n = 5$). The *IV* relation indicated with closed circles was obtained by shifting the *IV* relation from cell-attached experiments by 40 mV to the left.

side, the depolarization of the patch membrane increased the relative NP_o in a voltage-dependent manner. The reduction of Ca^{2+} concentration from 0.8 to 0.5 μM shifted the voltage-dependence of relative NP_o toward a positive direction. Thus, these channels showed both voltage- and Ca^{2+}-dependence.

The number of simultaneously open channels was 5.4 ± 0.7 per patch ($n = 5$) at 0 mV in the presence of 1 μM Ca^{2+}. Thus, the density of these channels were at least 6 channels per patch.

The current-voltage (*IV*) relation in the inside-out patch-clamp experiments described above is shown in Fig. 2. By using the Goldman, Hodgkin and Katz equation, permeability to K^+ ion (P_K) of these channels was calculated to be (5.4 ± 0.2) × 10^{-13} cm^3/sec ($n = 9$). Single-channel conductance was calculated from the P_K as 276 ± 8 pS ($n = 9$) in symmetric 140 mEq K^+ solution. Thus, there existed maxi K^+ channels in the apical membrane of the rabbit CNT.

Stretch-Activation of Maxi K^+ Channels

We examined stretch-activation of maxi K^+ channels in cell-attached configuration (Fig. 3). To augment the size of their current, we held a patch pipette potential (V_p) at −60 mV, i.e., the patch membrane was depolarized by 60 mV from an intrinsic apical membrane voltage (V_a). Under this patch membrane voltage, there should be enough driving force for Ca^{2+} influx via stretch-activated non-selective cation channels, although their current size was minimized.

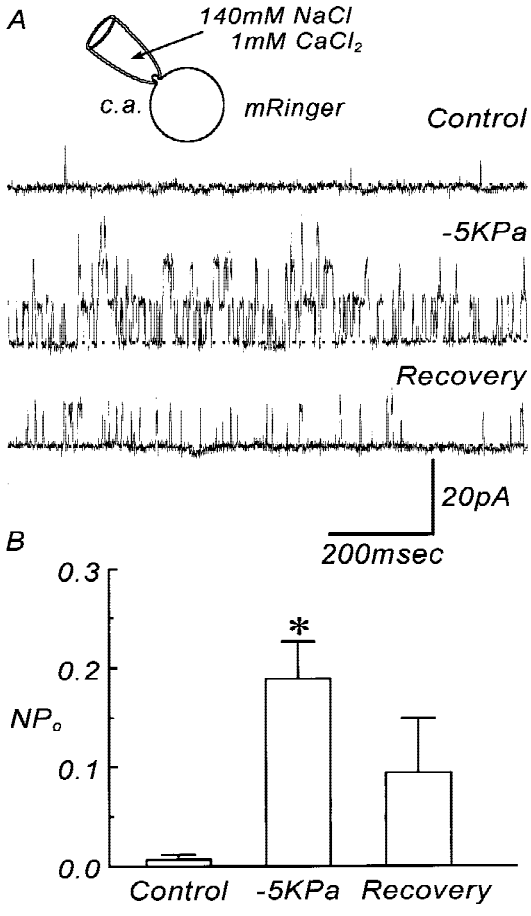

Fig. 3. Stretch-activation of maxi K^+ channels. (*A*) Single-channel currents were recorded from cell-attached (*c. a.*) patch at $V_p = -60$ mV in modified Ringer solution (mRinger). The pipette solution was the same as that described in Fig. 1. These pipette and bathing solutions were the same in all the cell-attached experiments otherwise mentioned. Negative pressure was applied to the patch pipette at −5 KPa, in order to stretch the patch membrane. (*B*) NP_o measured before, during, and after applying negative pressure in 7 experiments. NP_o in recovery period was measured at 1–1.5 min after stopping the application of negative pressure. *A significant change was observed compared with the preceding period.

Before stretching the membrane, few openings of maxi K^+ channels were observed ($NP_o = 0.007 \pm 0.004$, $n = 7$). This observation was very similar to that reported in the rat CCD (Wang et al., 1990). However, the NP_o was significantly increased to 0.189 ± 0.039 ($n = 7$), when the membrane was stretched by applying negative pressure (−5 KPa) to the patch pipette. The channel activity was gradually restored after releasing negative pressure. The NP_o measured during 1 to 1.5 min in the recovery period was 0.094 ± 0.055 ($n = 7$).

As shown in Fig. 2, only outward current was recorded during the stretching of the membrane. Thus, this current should be carried by the K^+ ion. Its *IV* relation plotted against $-V_p$ was very similar to that of maxi K^+

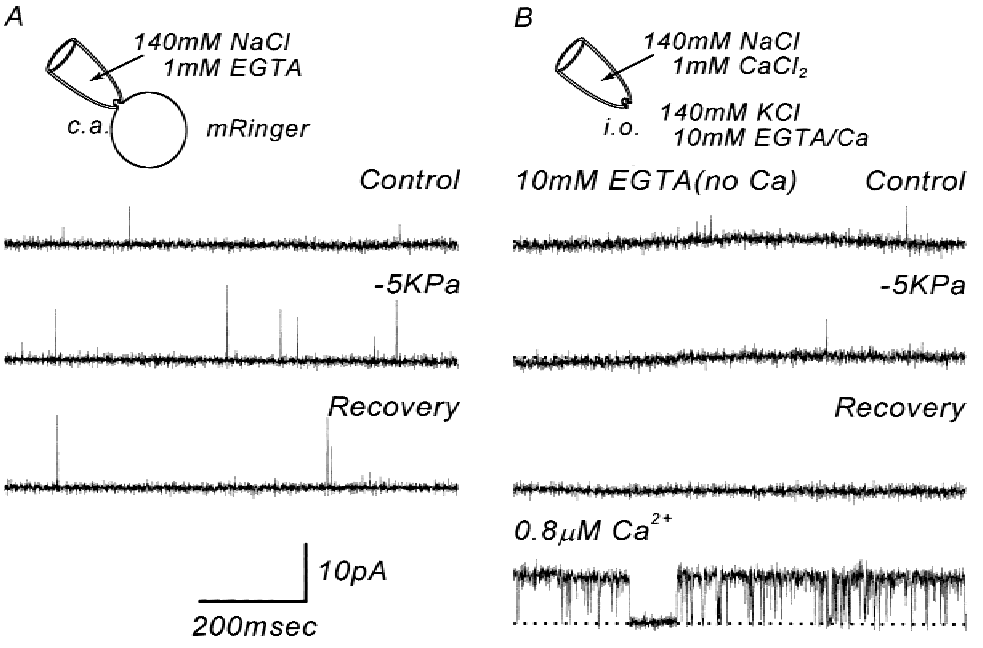

Fig. 4. Disappearance of stretch-activation of maxi K^+ channels. (*A*) Cell-attached experiments in which Ca^{2+} was omitted from and 1.0 mM EGTA was added into the patch pipette. No significant change in single-channel current recordings at $V_p = -60$ mV was observed before (top), during (2nd) and after applying negative pressure (3rd recording). (*B*) Inside-out patch-clamp experiments at $V_m = 0$ mV in which cytoplasmic Ca^{2+} was completely chelated by 10.0 mM EGTA before (top), during (2nd), and after applying negative pressure in patch pipette (3rd recording). The existence of maxi K^+ channels was verified by increasing the free Ca^{2+} concentration in the bathing solution to 0.8 μM in every 3 experiments (the lowest recording).

channel current recorded from inside-out patch membrane where the currents were plotted against V_m. This similarity of *IV* relation could be expected from the ionic condition employed in both experiments if the outward current is carried via maxi K^+ channels. In fact, the former *IV* relation could almost completely be superimposed on the latter one when the curve was shifted to the left by 40 mV (Fig. 2). Because no other large-conductance K^+ channels were found in excised patch-clamp experiments, the stretch-activated K^+ channel must be maxi K^+ channel.

We examined coupling between stretch-activated cation channels and maxi K^+ channels by two series of experiments (Fig. 4). As shown in Fig. 4*A*, we conducted the same type of experiment in cell-attached configuration except that Ca^{2+} was omitted from and 1 mM EGTA was added into the patch pipette. In contrast to the experiments conducted in the presence of 1 mM $CaCl_2$ in the pipette, no significant stretch-activation of maxi K^+ channels was observed ($NP_o = 0.002 \pm 0.002$ during the membrane stretch period *vs.* $NP_o = 0.003 \pm 0.002$ during the control period or $NP_o = 0.001 \pm 0.001$ during the recovery period, $n = 7$).

In another series of experiments (Fig. 4*B*), we added 10 mM EGTA into the cytoplasmic solution (bath solution) in inside-out configuration to remove the Ca^{2+} that was carried to the cytoplasmic side of patch membrane via stretch-activated cation channels. In this condition, no openings of maxi K^+ channels were recorded during control, membrane stretch, and recovery periods in which NP_o was less than 0.001 in 3 experiments. Existence of the channels was checked by increasing cytoplasmic free Ca^{2+} concentration to 0.8 μM ($NP_o = 0.575 \pm 0.112$, $n = 3$), as shown in the lowest recording. Thus, it is suggested that local Ca^{2+} entry via stretch-activated cation channels is necessary for the activation of maxi K^+ channels.

Effects of Inhibitors on Maxi K^+ Channels and Low-Conductance K^+ Channels

It is known that CTX blocks maxi K^+ channels in various tissues (Miller et al., 1985; Zweifach et al., 1991; Taniguchi, Furukawa & Shigekawa, 1993) and that AA inhibits low-conductance K^+ channels in the rat CCD (Wang, Cassola & Giebisch, 1992). Thus, we compared the inhibitory effects of these drugs on these two types of K^+ channels to examine which channels preferentially contributed to the K^+ conductance in the apical membrane of the rabbit CNT.

Figure 5*A* shows the blocking action of CTX on maxi K^+ channels in outside-out patch-clamp experiments. They were dose-dependently blocked by CTX

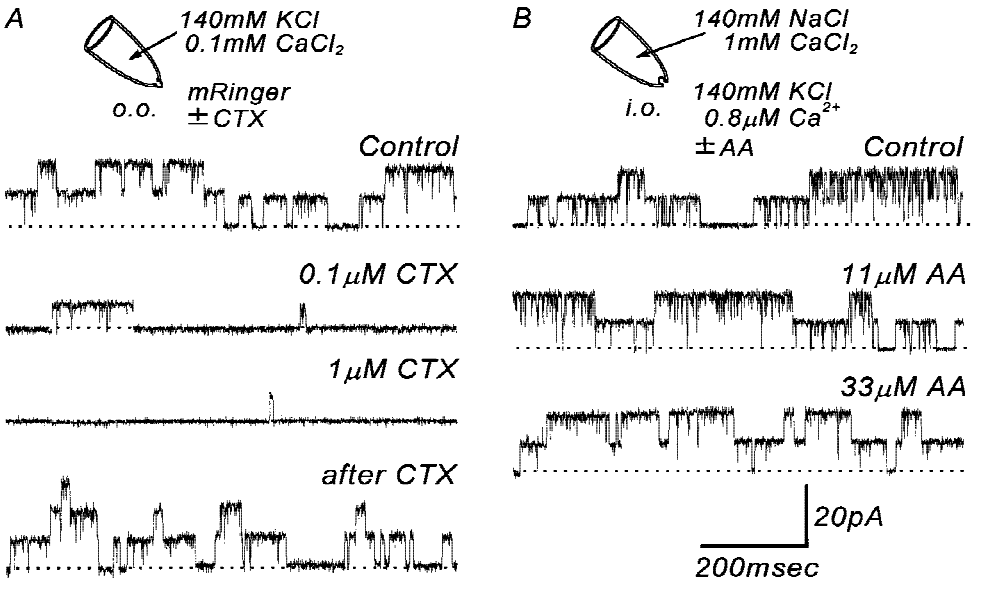

Fig. 5. Effects of charybdotoxin (CTX) and arachidonic acid (AA) on maxi K^+ channels. (*A*) Effect of CTX was examined in 4 outside-out (o. o.) patch-clamp experiments. Control NP_o was 0.531 ± 0.135 ($n = 4$). CTX was applied to the bathing solution. Pipette solution (in mM): 140.0 KCl, 0.1 $CaCl_2$, 10.0 HEPES/Tris, pH7.4. Bathing solution: modified Ringer solution. (*B*) Effect of AA was examined in 6 inside-out patch-clamp experiments. Control NP_o was 1.291 ± 0.426 ($n = 6$). AA was applied to bathing solution. Free Ca^{2+} concentration in the bathing solution was 0.8 μM. All the currents in *A* and *B* were recorded at 0 mV.

applied to the extracellular side of the patch membrane. Relative NP_o normalized by control NP_o was significantly decreased to 0.270 ± 0.044 ($n = 4$) at 0.1 μM and 0.028 ± 0.006 ($n = 4$) at 1 μM CTX, respectively. After removal of CTX, the relative NP_o was restored to 0.908 ± 0.283 ($n = 4$).

On the other hand, 11 μM AA applied to the cytoplasmic side of the patch membrane did not significantly change the maxi K^+ channel activity (relative NP_o = 1.151 ± 0.115, $n = 6$, Fig. 5*B*). No inhibitory effect was observed in relative NP_o (1.424 ± 0.284, $n = 6$), even after the concentration of AA was increased to 33 μM.

We also found low-conductance K^+ channels in the apical membrane of the rabbit CNT in outside-out patch-clamp experiments. The K^+ permeability of single low-conductance K^+ channel obtained from *IV* relation (*not shown*) was (6.9 ± 0.8) × 10^{-14} cm^3/sec ($n = 5$), which gave their single-channel conductance of 35 ± 3 pS ($n = 5$) in symmetric 140 mEq K^+ solution. This value was very similar to that reported in the rat CCD (Wang et al., 1990).

As shown in Fig. 6*A*, the activity of low-conductance K^+ channels recorded from the outside-out patch membrane was not significantly diminished even in the presence of extracellular 1 μM CTX (relative NP_o = 1.051 ± 0.052, $n = 5$). The patch membrane polarity was checked by adding 1 mM ATP to the bathing solution, because the ATP was known to inhibit their activity from the cytoplasmic side (Wang et al., 1990). No significant reduction of relative NP_o (1.063 ± 0.072, $n = 5$) was observed after applying ATP; i.e., the patch membrane should be outside-out.

On the contrary, the low-conductance K^+ channels were completely inactivated by 11 μM AA applied to the cytoplasmic side in inside-out configuration (relative NP_o = 0.024 ± 0.014, $n = 5$) (Fig. 6*B*), as reported in the rat CCD (Wang et al., 1992).

Effects of Luminal Fluid Flow and K^+ Channel Inhibitors on Apical K^+ Conductance

When K^+ concentration in the lumen is abruptly increased, the apical membrane may be depolarized, reflecting the K^+ conductance of the apical membrane. The change, in turn, is associated with negative deflection of V_t. Thus, the deflection of V_t (ΔV_t) in response to an increment of luminal K^+ concentration may represent the apical K^+ conductance.

Figure 7 shows the luminal flow-dependence of apical K^+ conductance in the rabbit CNTs perfused in vitro. In these experiments, luminal K^+ concentration was increased from 5 to 15 mEq by adding 10 mM K gluconate in the luminal solution. The ΔV_t was measured as maximal deflection of V_t during an application of 15 mEq K^+ solution from a line extrapolated from basal V_t prior to K^+ step. Thus, a negative value of ΔV_t shows negative shift of V_t.

After 1–3 hr equilibration with low perfusion pressure (0.5 KPa), the V_t reached a voltage more negative

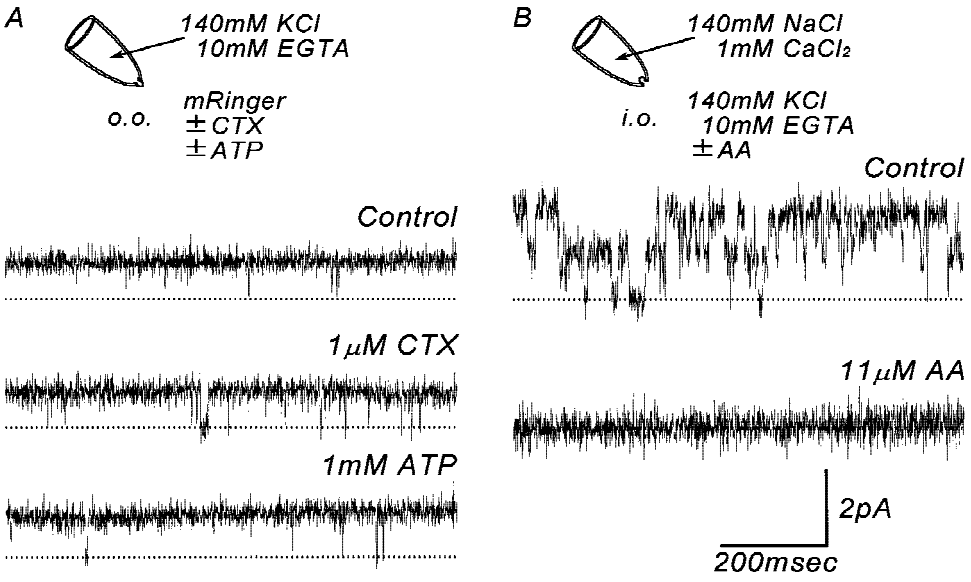

Fig. 6. Effects of CTX and AA on low-conductance K^+ channels. (*A*) Effect of CTX was examined in 5 outside-out patch clamp experiments. Control NP_o was 1.781 ± 0.253 ($n = 5$). Polarity of patch membrane was checked by applying 1.0 mM ATP in the bathing solution. (*B*) Effect of AA was examined in 5 inside-out patch clamp experiments. Control NP_o was 1.381 ± 0.230 ($n = 5$). Experimental conditions were the same as described in Fig. 5 except that $CaCl_2$ was omitted from the pipette solution (*A*) and from the bathing solution (*B*), to suppress the activity of maxi K^+ channels. All the currents in *A* and *B* were recorded at 0 mV and pH7.4.

than −30 mV. To obtain an accurate ΔV_t, we changed the luminal K^+ concentration while the V_t was stable. The V_t measured just before a luminal application of K gluconate was −45.1 ± 2.6 mV ($n = 6$). As shown in upper left panel of Fig. 7*A*, little negative shift of V_t was recorded in response to a 3-fold increase of luminal K^+ concentration ($\Delta V_t = -0.7 \pm 0.4$ mV, $n = 6$).

Increasing perfusion pressure from 0.5 to 1.5 KPa stabilized the V_t at a less negative voltage ($V_t = -13.3 \pm 2.9$ mV, $n = 6$, just before an increment of luminal K^+ concentration). In this condition, ΔV_t was significantly increased to −9.4 ± 0.9 mV ($n = 6$) (Fig. 7*A*, upper right panel). These observations suggested the flow-dependence of apical K^+ conductance, although the V_t should be affected by the pressure-dependent change of mechanical leakage of the tubule as well as the change of ionic conductances. A reduction of K^+ conductance rather than an increment of Na^+ conductance in apical membrane, therefore, might contribute to a generation of huge negative V_t, when the tubule was perfused with lower pressure.

In the above experiments, however, luminal K^+ concentration might have been already increased to about 15 mEq due to K^+ secretion, when the tubule was slowly perfused with lower perfusion pressure. Thus, the V_t might not respond to the increment of luminal K^+ concentration to 15 mEq. In another series of experiments, we increased luminal K^+ concentration from 5 to 50 mEq by replacing equivalent Na^+, to minimize the effect of K^+ accumulation in the tubular lumen. In these experiments, the ΔV_t (−23.2 ± 5.7 mV, $n = 4$) during higher perfusion pressure period was also significantly larger than that (−4.3 ± 2.1 mV, $n = 4$) during lower perfusion pressure period. The possibility described above could be ruled out by these observations.

A luminal application of 1 μM CTX shifted the V_t to a more negative voltage ($V_t = -25.1 \pm 5.1$ mV, $n = 6$, just before increasing luminal K^+ concentration). The value of V_t, however, was not accurate, because the CTX made the V_t unstable. We conducted the K^+ step experiment during a relatively stable period in this condition. In the presence of CTX, the ΔV_t in response to an increase of luminal K^+ concentration to 15 mEq was significantly decreased to −1.3 ± 0.6 mV ($n = 6$) (Fig. 7*A*, lower left panel) compared with that observed in the preceding period. The recovery from the effect of CTX ($\Delta V_t = -3.8 \pm 1.8$ mV, $n = 6$) (Fig. 7*A*, lower right panel) was not significant probably due to an incomplete removal of the toxin. These observations suggested that the deflection of V_t mainly originated from CTX-sensitive K^+ conductance rather than the K^+ diffusion voltage via paracellular pathway.

Contrary to the effect of CTX, a luminal application of 33 μM AA did not significantly change the ΔV_t recorded from the CNT that was perfused at 1.5 KPa (Fig. 8), although this concentration of AA completely inhibited the low-conductance K^+ channel activity (Fig. 6*B*). The ΔV_t during the control period and during the application of the drug and recovery periods was −7.2 ± 0.5, −6.7 ± 0.6 and −5.9 ± 1.0 mV, respectively, in 5 tubules.

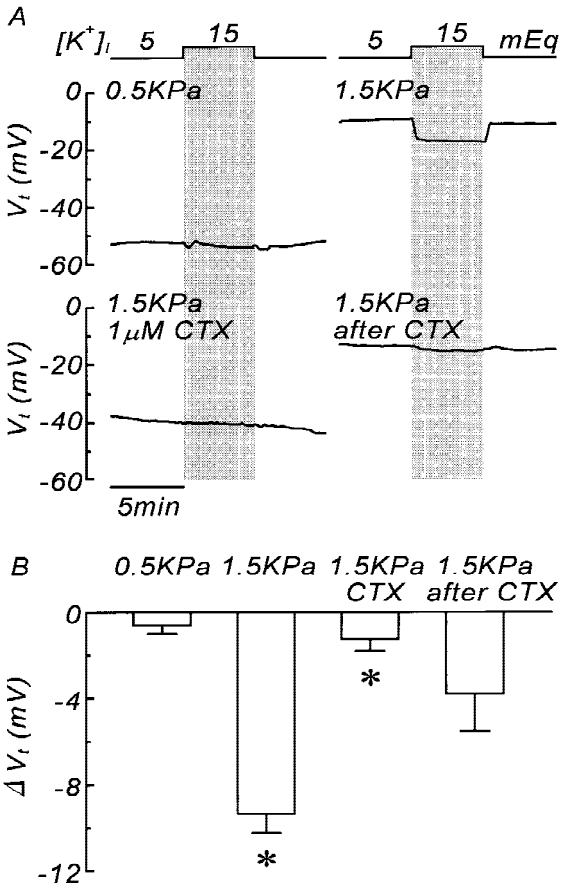

Fig. 7. Effect of CTX on flow-dependence of apical K^+ conductance. (*A*) Apical K^+ conductance was estimated from ΔV_t in response to an addition of 10.0 mM K gluconate into the luminal solution containing 5.0 mM KCl of 6 rabbit CNTs which were perfused by applying hydraulic pressure of 0.5 or 1.5 KPa. CTX was applied into the lumen to examine the contribution of maxi K^+ channels to apical K^+ conductance. (*B*) Summary of the estimated apical K^+ conductance in each condition in *A*. *Significant change was observed compared with the preceding period.

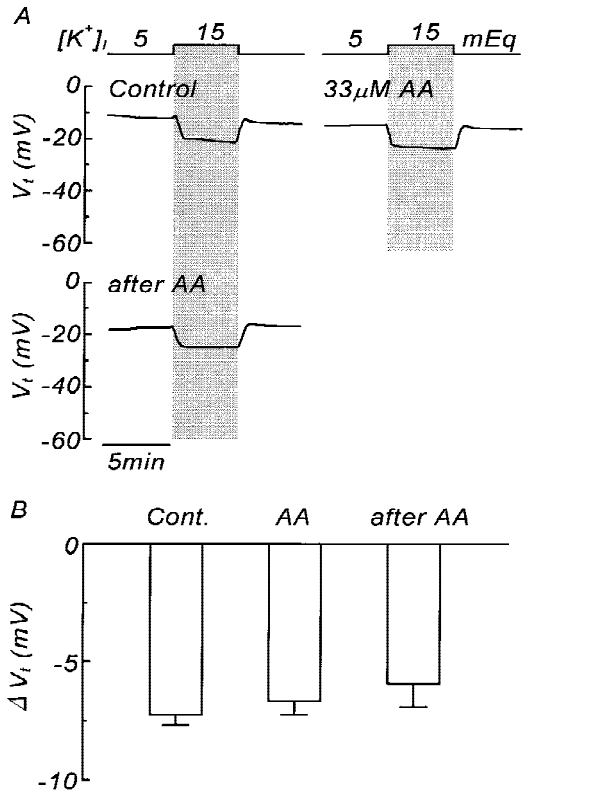

Fig. 8. Lack of AA effect on the apical K^+ conductance. (*A*) Apical K^+ conductance was estimated with the same way described as in Fig. 7 in 5 rabbit CNTs. AA was applied into the lumen to examine the contribution of low-conductance K^+ channels to apical K^+ conductance. (*B*) Summary of the estimated apical K^+ conductance in each condition in *A*.

The comparison between the effects of CTX and AA on the ΔV_t suggested that the major part of flow-dependent apical K^+ conductance was constituted by a CTX-sensitive component, i.e., maxi K^+ channels, in the rabbit CNT.

Discussion

Secretory Pathway for K^+

It was reported that the increased secretion of K^+ along the distal nephron in response to acute K^+ loading was augmented by a chronic high K^+ diet (Stanton & Giebisch, 1982). This finding was supported by the morphological observations that the high K^+ diet induced hypertrophy in association with augmented infoldings of the basolateral membrane in both CNT and CD (collecting duct) cells in both rat (Stanton et al., 1981) and rabbit kidneys (Kaissling & Le Hir, 1982). These observations suggest that the CNT and CCD are responsible for the distal K^+ secretion.

The activity of maxi K^+ channels seems to be very low in cell-attached patch-clamp experiments, because a rather high concentration of cytoplasmic Ca^{2+} is necessary for their activation. However, their open probability and density per patch membrane were not small in the rabbit CNT, when the patch membrane was stretched by negative pressure applied to the patch pipette. In our previous study (Taniguchi et al., 1994), we reported in the rabbit CNT that an elevation of luminal fluid flow augmented the apical Ca^{2+} influx via stretch-activated cation channels. Along with these observations, we found in the present study that apical K^+ conductance was stimulated flow-dependently by increasing perfusion pressure to 1.5 KPa.

This perfusion pressure seems to be slightly higher than the luminal pressure measured in the normal rat distal nephron which oscillates between 0.8 and 1.1 KPa (Holstein-Rathlou & Marsh, 1989). But mean luminal pressure in the distal nephron was transiently raised to 2.4 KPa and reached 1.3 KPa in steady state by intravenously applying a low dose (1.67 mg/Kg) of acetazolamide, a mild diuretic (Leyssac, Karlsen & Skøtt, 1991). Thus, the perfusion pressure used in our experiments must be in a reasonable physiological range of luminal pressure in the distal nephron.

Mean concentration of cytoplasmic Ca^{2+} measured with fura-2 in the previous work (Taniguchi et al., 1994) might not be high enough to activate maxi K^+ channels on the basis of their Ca^{2+} dependence. Nevertheless, the stretch-activation of maxi K^+ channels should be Ca^{2+} dependent, because the maxi K^+ channels did not show any evident stretch-activation by themselves in the absence of ambient Ca^{2+}. This was confirmed in two different series of experiments, i.e., cell-attached patch-clamp experiments in which Ca^{2+} was omitted from the patch pipette (Fig. 4*A*) and inside-out patch clamp experiments in which Ca^{2+} in the cytoplasmic side was completely chelated by EGTA (Fig. 4*B*). Thus, it is possible that the local Ca^{2+} concentration just under the apical membrane, where stretch-activated cation channels are located, is much higher than the mean Ca^{2+} concentration in cytoplasm.

In addition, a luminal application of CTX, which blocked maxi K^+ channels in the apical membrane of the CNT, significantly decreased this flow-dependent apical K^+ conductance, whereas the apical K^+ conductance was not affected by luminal application of AA which inactivated low-conductance K^+ channels. These observations strongly support the view that the maxi K^+ channels work as a dominant apical pathway for K^+ secretion in the presence of physiological luminal fluid flow at least in the rabbit CNT. Although one may argue that the negative data for AA may be simply explained by the view that AA acts only from the cytoplasmic side. However, Wang and Lu (1995) reported that AA inhibited 70 pS K^+ channel in the apical membrane of rat thick ascending limb in either inside-out or cell-attached configuration.

In contrast, it seems that the maxi K^+ channels do not play a dominant role in the K^+ secretion of the CCD, because of their small open probability and rare incidence in cell-attached configuration, compared with those of low-conductance K^+ channels (Wang et al., 1990). Furthermore, whole-cell current was not sensitive to TEA which was known to block the maxi K^+ channels in CD cell of the rat, whereas large TEA-blockable current was recorded from intercalated cells dialyzed with 10 μM Ca^{2+} (Pácha et al., 1991). This strong evidence seems to establish the above view, because the CD cell is considered to be responsible for K^+ secretion along CCD (Stanton et al., 1981; Kaissling & Le Hir, 1982).

On the other hand, Hirsch et al. (1993) reported in the rat CCD that the maxi K^+ channel was totally inactivated by applying 5 mM ATP to the cytoplasmic side of the patch membrane in inside-out patch-clamp experiments, although the type of patch-clamped cell was not identified. However, these maxi K^+ channels must be located in the CD cells, because Pácha et al. (1991) recorded the TEA-sensitive whole-cell current from the intercalated cells by using pipettes containing 10 mM ATP. If this is true, the maxi K^+ channels in the CD cells should be inactivated by the ATP in their experiments. Thus, the role of maxi K^+ channels in K^+ secretion along the CCD cannot be still ruled out.

Furthermore, it has been reported in several tissues (Gray et al., 1990; Klærke et al., 1996; Moreau et al., 1996) that the activity of maxi K^+ channels is markedly stimulated by adenosine 3′, 5′-cyclic monophosphate (cAMP) dependent protein kinase (protein kinase A). Because several hormones like parathyroid hormone (PTH) or vasopressin are known to stimulate cAMP cascade in the distal nephron segments (Morel, Imbert-Teboul & Chabardés, 1981), it is likely that the activity of maxi K^+ channels in vivo is higher than those observed in the previous (Frindt & Palmer, 1987; Hirsch et al., 1993) and our present studies. The maxi K^+ channels should be investigated in more physiological condition including luminal fluid flow and hormone stimulation, in order to explore their role in the distal K^+ secretion.

Flow-Dependent Secretion of K^+

It is well known that K^+ excretion rate is accelerated by diuretics like furosemide or thiazide which increase fluid delivery to the distal nephron, although their primary action is an inhibition of NaCl transport (Wright & Giebisch, 1992). The acceleration of K^+ excretion has been explained by augmented K^+ secretion along the distal nephron, which is proportional to the fluid delivery (Khuri et al., 1975; Good & Wright, 1979). Good and Wright (1979) proposed a hypothesis that an increased luminal fluid flow preserved driving force for K^+ secretion, because they found the reduction of K^+ secretion by increasing luminal K^+ concentration. Their hypothesis, however, is not necessarily contradictory to the hypothesis of flow-dependent apical K^+ conductance in the distal nephron segments. The latter is very consistent with our finding that the apical K^+ conductance produced by maxi K^+ channels was dependent on luminal fluid flow by coupling with Ca^{2+}-permeable stretch-activated cation channels. In support of the latter hypothesis, Okusa et al. (1991) reported that when using in vivo microper-

fusion technique the relationship between V_t and luminal flow rate was dependent on luminal Ca^{2+} concentration in the rat distal nephron in the presence of luminal amiloride.

According to the latter hypothesis, however, both increased flow rate of luminal fluid and luminal Ca^{2+} concentration not only cause an increased apical K^+ conductance for K^+ secretion but also cause hyperpolarization of the apical membrane, which reduces the driving force for the K^+ secretion. The observation (Okusa et al., 1990) that an addition of physiological concentration of luminal Ca^{2+} suppressed distal K^+ secretion may be explained by the hyperpolarization, because it was observed at the luminal flow rate of 15 nl/min where the negative V_t was minimized by luminal Ca^{2+} (Okusa et al., 1990, 1991). Nevertheless, the effect of luminal Ca^{2+} on distal K^+ secretion was equivocal, because the amount of K^+ secretion measured in the distal nephron of the same animal by using in vivo micropuncture technique was linearly correlated with the flow rate of luminal fluid up to 30 nl/min (Khuri et al., 1975) in the presence of similar concentration of Ca^{2+} in the lumen (Vick & Costanzo, 1988).

It is known that the amount of K^+ excretion into urine was not increased during water diuresis (Evans et al., 1954). This is contradictory to the flow-dependent K^+ secretion along distal nephron segments, because luminal flow rate must be increased in these segments during water diuresis. Field, Stanton and Giebisch (1984), however, dissolved the contradiction by demonstrating in rat kidney with in vivo micropuncture and microperfusion techniques that vasopressin stimulated the K^+ secretion along distal nephron. According to their view, the accelerating effect of luminal flow on K^+ secretion, probably from CNT, might be cancelled by the decelerating effect of reduced plasma vasopressin level on K^+ secretion from CCD, a target of vasopressin, during water diuresis. Thus, the amount of K^+ excretion into urine could be maintained at a similar level in either antidiuresis or water diuresis.

Role of Maxi K^+ Channels in Ca^{2+} Transport

Ca^{2+} transport is stimulated by PTH which mainly acts on the CNT in the rabbit kidney (Shimizu et al., 1990*b*). Taniguchi et al. (1994) reported in the rabbit CNT that PTH stimulated the activity of Ca^{2+}-permeable stretch-activated cation channels via formation of cAMP. The cation influx depolarized the apical membrane of CNT which was suggested by the observation that V_t shifted negatively in the initial phase of PTH effects (Shimizu et al., 1990*a*). The depolarization of the apical membrane would reduce the driving force for Ca^{2+} influx.

However, this inefficiency for Ca^{2+} reabsorption could be restored by the stimulation of Ca^{2+}-activated maxi K^+ channels as well as the inactivation of amiloride-sensitive Na^+ channels (Shimizu et al., 1990*a*), both of which should hyperpolarize the apical membrane of the CNT. In fact, it was reported with stop-flow study in the rabbit (Tabei et al., 1984) and in vivo micropuncture technique in the rat (Baily, Roinel & Amiel, 1985) that the PTH stimulated K^+ secretion as well as Ca^{2+} reabsorption along the distal nephron. The hyperpolarization of the apical membrane may be accelerated by the phosphorylation of maxi K^+ channels, because PTH is known to stimulate maxi K^+ channel activity by stimulating protein kinase A in osteoblast bone cell (Moreau et al., 1996).

Conclusion

The Ca^{2+}-activated maxi K^+ channel is a dominant K^+ channel of the apical membrane of the CNT. This channel would play a major role in the K^+ secretion in the CNT, representing flow-dependent secretion of K^+. In combination with flow-dependent activation of the nonselective cation channel in the apical membrane of this segment, the maxi K^+ channel in the CNT may play a critical role in the mutual interaction between K^+ secretion and Ca^{2+} absorption regulated by PTH.

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