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Pressure- and Parathyroid-Hormone-dependent Ca^{2+} Transport in Rabbit Connecting Tubule: Role of the Stretch-activated Nonselective Cation Channel

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Abstract. To characterize the Ca^{2+} transport process across the apical membrane of the rabbit connecting tubule (CNT), we examined the effects of luminal pressure on parathyroid hormone (PTH)-dependent apical Ca^{2+} transport in this segment perfused in vitro. An increase of perfusion pressure (0.2 to 1.2 KPa) caused cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) to increase by 42 ± 11 nM in Fura-2 loaded perfused CNT. The response was accentuated when 10 nM PTH was added to the bath (101 ± 30 nM, $n = 6$). Addition of 0.1 mM chlorphenylthio-cAMP (CPT-cAMP) to the bath also augmented the $[Ca^{2+}]_i$ response to pressure from 36 ± 16 to 84 ± 26 nM ($n = 3$). Under steady perfusion pressure at 1.2 KPa, PTH (10 nM) increased $[Ca^{2+}]_i$ by 31 ± 7 nM ($n = 5$), whereas it did only slightly by 6 ± 2 nM ($n = 12$) at 0.2 KPa. The pressure-dependent increase of $[Ca^{2+}]_i$ was abolished by removing luminal Ca^{2+} ($n = 3$), and was not affected by 0.1 and 10 μM nicardipine ($n = 4$) in the presence of 10 nM PTH. Cell-attached patch clamp studies on the apical membrane of everted CNT with pipettes filled with either 200 mM $CaCl_2$ or 140 mM NaCl revealed channel activities with conductances of 42 ± 2 pS ($n = 4$) or 173 ± 7 pS ($n = 5$), respectively. An application of negative pressure (−4.9 KPa) to the patch pipette augmented its mean number of open channels (NP_o) from 0.005 ± 0.001 to 0.022 ± 0.005 in the Ca^{2+}-filled pipette, and was further accelerated to 0.085 ± 0.014 ($n = 3$) by 0.1 mM CPT-cAMP. In the Na^+-filled pipette, similar results were obtained ($n = 3$), and CPT-cAMP did not activate the stretch-activated channel in the absence of negative pressure ($n = 3$). These results suggest that a stretch-activated nonselective cation channel exists in the apical membrane of the CNT and that it is activated by PTH in the presence of hydrostatic pressure, allowing entry of Ca^{2+} transport from the apical membrane.

Key words: Distal nephron — Stretch-activated channel — Nonselective cation channel — Patch clamp study — Cytosolic free Ca^{2+}

Introduction

Parathyroid hormone (PTH) plays an important role in the Ca^{2+} homeostasis (Suki & Rouse, 1991). In the rabbit kidney, the PTH-sensitive adenylate cyclase has been shown to be distributed in various nephron segments including the proximal convoluted (PCT) and straight (PST) tubules, the cortical thick ascending limb of Henle's loop (CAL), and the connecting tubule (CNT) (Chabardès et al., 1975). In vitro microperfusion studies demonstrated that PTH stimulates Ca^{2+} transport most prominently in the CNT (Shareghi & Stoner, 1978; Imai, 1981) and moderately in the CAL (Imai, 1981). A series of the subsequent studies confirmed the notion that the CNT is the most important site of Ca^{2+} transport regulated by PTH (Bourdeau & Lau 1989; Lau & Bourdeau, 1989; Bourdeau & Eby, 1990; Shimizu et al., 1990*a,b*, 1991).

Bourdeau and Lau (1989) observed that in the in vitro perfused rabbit CNT, PTH increases cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) as measured by the microscopic fluorometry, indicating that Ca^{2+} entry across the apical membrane is an initial step for the stimulation of Ca^{2+} transport by PTH. However, mechanisms of Ca^{2+} entry across the apical membrane of the CNT have not been well established. Based on the biphasic response of the apical membrane voltage to PTH, Shimizu et al. (1990*a*) proposed a hypothesis that

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a nonselective cation channel might be responsible for the entry of Ca^{2+} across the apical membrane. A more direct evidence in support of this notion was provided by Taniguchi, Yoshitomi and Imai (1992), who demonstrated a stretch-activated nonselective cation channel by patch clamp on the apical membrane of the everted CNT. On the other hand, in the epithelia derived from the distal straight or convoluted tubules, several investigators have proposed that Ca^{2+} channels are responsible for the entry of Ca^{2+} from the apical membrane (Bacskai & Friedman, 1990; Lau, Quamme, & Tan, 1991; Poncet, Merot, & Poujeol, 1992).

Apart from the issue mentioned above, the magnitude of Ca^{2+} transport stimulated by PTH has been reported to be considerably discrepant between two groups of investigators: the values reported by our group (Imai, 1981; Shimizu et al., 1990*a*, 1991) were 5–6 times higher than those reported by Shareghi and Stoner (1978). The major difference in experimental conditions between these studies was a marked difference of perfusion rates: the former group used high perfusion rates (10–20 nl/min), whereas the latter used very low flow rates (less than 5 nl/min). This issue, in conjunction with the finding of stretch-activated nonselective cation channels in the apical membrane of the CNT (Taniguchi et al., 1992), lead us to speculate that the Ca^{2+} transport stimulated by PTH in the CNT is critically dependent on the luminal perfusion pressure. The present study, therefore, was designed to test this hypothesis by observing effects of perfusion pressure on $[Ca^{2+}]_i$ stimulated by PTH by using microscopic fluorometry and the patch clamp technique to characterize the stretch-activated nonselective cation channel in detail. We demonstrated that the stretch-activated nonselective cation channel plays a key role for the pressure- or flow-dependent PTH effect on Ca^{2+} reabsorption as well as the basal Ca^{2+} transport in the CNT.

Materials and Methods

Preparation of the CNT

The CNTs from rabbit kidneys were isolated and perfused according to the method of Burg et al. (1966) as modified previously (Shimizu et al., 1990*a,b*). In brief, the kidney was removed from Japanese white rabbits of either sex weighing 2.0–2.5 kg which were anesthetized with pentobarbital sodium (35 mg/kg iv). After kidney slices were placed 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, pH 7.4, CNT was isolated at 4°C with fine forceps according to the criteria reported previously (Imai, 1979).

For Ca^{2+} measurement, the tubule was hooked up to a set of perfusion pipettes equipped on a microperfusion apparatus (Narishige, Tokyo), and perfused with the solution containing (in mM): 135.0 NaCl, 5.0 KCl, 1.0 NaH_2PO_4/Na_2HPO_4, 5.0 $CaCl_2$, 1.0 $MgCl_2$, 5.6 glucose, 5.0 alanine, and 10.0 *N*(2-hydroxyethyl)-piperazine-*N'*-2 ethanesulfonic acid (HEPES), of which pH was adjusted to 7.4 by adding tris(hydroxymethyl)amino-methane (Tris). Ca^{2+} was eliminated from the bathing fluid. Perfusion pressure was varied by changing the height of the fluid reservoir connected with the inner perfusion pipette, which was filled with luminal solution. The outflow of the perfused tubule was loosely folded with a collection pipette whose inner diameter was about two times larger than the outer diameter of the CNT, so that we may avoid any increases in resistance at the tubular outflow.

For patch clamp experiments, the CNT was everted with microperfusion pipettes in the bathing solution containing 1.8 mM $CaCl_2$, as described by Engbretson, Beyenbach and Stoner (1988). Briefly, the tubule was aspirated into an outer perfusion pipette. The end of the tubule was picked up with an inner perfusion pipette, then the pipette was advanced with applying suction to the outer pipette. The tubule was everted by this procedure, so that we could approach the apical membrane with a patch pipette without touching any other portions of the tubule. We did not pull out the inserted inner perfusion pipette from the everted tubule to avoid serious damages to the CNT. Thus, the basolateral side of the tubule was not perfused. The existence of the inner pipette in the everted basolateral side was also convenient to avoid the movement of the tubular preparation caused by flow turbulence.

Ca^{2+} Measurement

The $[Ca^{2+}]_i$ of microperfused CNT was measured with Fura-2 (Grynkiewicz, Poenie & Tsien, 1985; Tsien, Rink & Poenie, 1985), generally as described by Hanaoka et al. (1993). An aliquot of the stock solution (1 mM in dimethylsulfoxide) of the acetoxymethyl ester of Fura-2 (Fura-2/AM) was added to the bath containing Ca^{2+}-free bathing solution (final concentration = 30 μM), and the tubule was incubated for 20 min at room temperature. The Fura-2 loaded CNT was rinsed by perfusing the bath with a Ca^{2+}-free bathing solution at 36°C for 5–10 min, then $[Ca^{2+}]_i$ was measured with a microscopic fluorescence photometry system (OSP-3, Olympus, Tokyo, Japan).

We set a large diameter of the beam focused on the tubule (75 μm), which exceeded the diameter of the CNT, to minimize the effect of tubular movement when luminal pressure was changed. The fluorescent dye was excited at 340 and 380 nm light supplied from a 75 W Xenon lamp. The fluorescence emission was measured at 510 nm. Emission signals excited at 340 nm (F_{340}) and at 380 nm (F_{380}) were sampled every 10 msec, then 100 signals were averaged to get one data point every second. Before calculating the fluorescence ratio ($R = F_{340}/F_{380}$), the background autofluorescence at each wavelength was subtracted from each emission signal. A neutral density filter (ND6W18, Olympus, Tokyo) was used to equalize the emission light.

Calibration curve was obtained from the following equation:

$$[Ca^{2+}]_i = K_d \frac{F_{min}(R - R_{min})}{F_{max}(R - R_{max})}, \quad (1)$$

where R_{min} and R_{max} were the fluorescence ratio at zero Ca^{2+} or at saturated Ca^{2+} concentration. F_{min} and F_{max} were the fluorescences excited by 380 nm light at zero Ca^{2+} or at saturated Ca^{2+} concentration. We used a value of 224 nM for the effective dissociation constant (K_d) of Fura-2 against Ca^{2+}. R_{min} and R_{max} were measured as 0.47 and 15.97, respectively, in the presence of 1 mM ethyleneglycol-bis(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) or 1 mM $CaCl_2$. F_{min}/F_{max} was measured as 28.91. However, the absolute value of $[Ca^{2+}]_i$ cannot be accurately calibrated, because the K_d should be changed in the presence of protein. Thus, we should note that the

calibrated values for $[Ca^{2+}]_i$ provide only relative changes in Ca^{2+} concentration.

To examine the effects of 1-34 human PTH (Peptide Institute, Osaka, Japan) and 8-(4-chlorphenylthio)-adenosine-cyclic 3',5'-monophosphate sodium (CPT-cAMP) (Boehringer Mannheim, FRG), we added 10 nM PTH or 0.1 mM CPT-cAMP to the Ca^{2+}-free bathing solution. In some experiments, $CaCl_2$ was omitted from the luminal solution and either 0.1 mM EGTA or nicardipine HCl (0.1 or 10 μM) was added.

Electrical Recordings

The cell-attached patch clamp (Hamill et al., 1981) was performed on the apical (originally luminal) membrane of the everted CNTs with an amplifier (L/M EPC-7, List Electronic, Darmstadt, FRG). Patch pipettes (3–5 MΩ) were filled with the solution containing (in mM): 140.0 NaCl, 10.0 HEPES/Tris, pH 7.4 (Na^+ pipette), 140.0 Na gluconate, 10.0 HEPES/Tris, pH 7.4 (Cl^- free Na^+ pipette) or 200.0 $CaCl_2$, 10.0 HEPES/Tris, pH 7.4 (Ca^{2+} pipette). To stretch the patch membrane, negative pressure, which was monitored with a hydraulic manometer, was applied in the patch pipette. The outward current from the cytoplasmic face into the pipette was described upwardly and positively, and inward current, vice versa.

Membrane voltage (V_m) of the everted CNT cells was measured at 36°C with a current clamp amplifier (Duo 773, WPI Instruments, New Haven, CT) by using a microelectrode filled with 0.5 M KCl. Because V_m of the CNT cells was measured to be -64.1 ± 2.5 mV ($n = 10$), patch membrane voltage (V_a) could be estimated as $V_m - V_p$ in cell-attached patch clamp experiments, where V_p was the pipette potential.

Current and voltage recordings were stored with an FM tape recorder (XR30, TEAC, Tokyo, Japan). The current recordings were filtered at the cutoff frequency (f_c) of 2 or 5 KHz with a 4-pole Bessel low-pass filter (E-3201A, NF Electronic Instruments, Yokohama, Japan), then digitized at 10 KHz with a TL-1 DMA interface (Axon Instruments, Foster City, CA). We analyzed them with pCLAMP software (Axon Instruments). The mean number of open channels (NP_o) was obtained from the current recordings during 30 sec or every second (for time course analysis).

Statistical Analysis

Statistical data were described as mean ± SE (n = number of experiments), and statistical significance was determined by Student's t-test for paired or nonpaired samples when appropriate. The P value less than 0.05 was considered to be significant.

Results

Effect of Luminal Pressure on Baseline or PTH-stimulated $[Ca^{2+}]_i$

To examine whether Ca^{2+} transport in the CNT is influenced by the luminal perfusion pressure, we observed $[Ca^{2+}]_i$ by changing the height of the fluid reservoir connected to the perfusion pipette in the presence or absence of PTH or CPT-cAMP. Figure 1 depicts representative tracings of $[Ca^{2+}]_i$. In the absence of PTH in the bath, an increase in perfusion pressure from 0.2 to 1.2 KPa caused a sharp increase in $[Ca^{2+}]_i$, which was reversed by decreasing perfusion pressure to the control level (Fig. 1*A*). We noted that the recovery of $[Ca^{2+}]_i$ tended to be gradual as opposed to the sharp increase of onset. An addition of 10 nM PTH to the bath caused a very gradual and small increase in $[Ca^{2+}]_i$ at low perfusion pressure. In the presence of PTH, an increase in perfusion pressure caused a sharp increase in $[Ca^{2+}]_i$ with the magnitude being greater than that observed in the absence of PTH. The results of six experiments showed that the increments of $[Ca^{2+}]_i$ upon an increase in perfusion pressure were 42 ± 11 and 101 ± 30 nM in the absence or presence of PTH, respectively. These values were significantly different ($P < 0.05$). The similar result was observed in the presence or absence of CPT-cAMP in the bath (Fig. 1*B*). The results of three experiments showed that the increments of $[Ca^{2+}]_i$ in response to increased perfusion pressure were 36 ± 16 and 84 ± 26 nM in the absence or presence of CPT-cAMP, respectively. The values were significantly different ($P < 0.05$).

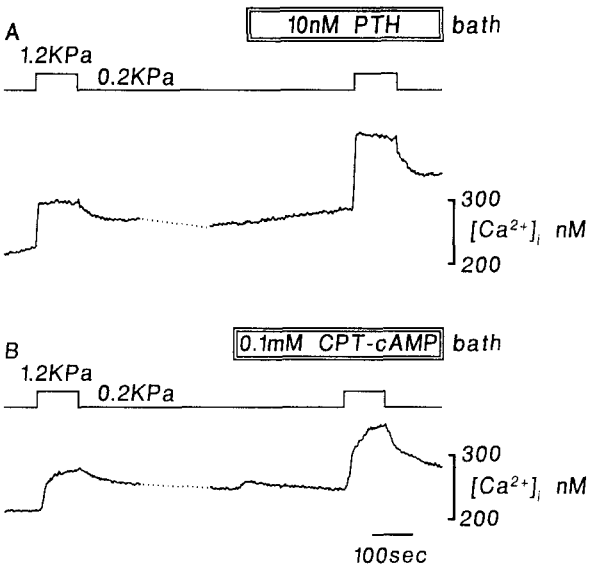

Fig. 1. Representative tracings of $[Ca^{2+}]_i$ in perfused CNT showing the effects of perfusion pressure on $[Ca^{2+}]_i$ in the presence or absence of PTH or CPT-cAMP. The rise of $[Ca^{2+}]_i$ in response to the increase in perfusion pressure from 0.2 to 1.2 KPa was exaggerated in the presence of 10 nM PTH in the bath (*A*). PTH increased basal $[Ca^{2+}]_i$ only slightly when the tubule was perfused at low pressure. Similarly, 0.1 mM CPT-cAMP in the bath (*B*) also augmented the pressure-dependent rise of $[Ca^{2+}]_i$ and caused only a slight increase in basal $[Ca^{2+}]_i$.

The repeated challenge of pressure application sometimes blunted the responses of $[Ca^{2+}]_i$ to hydrostatic pressure. In the absence of PTH or CPT-cAMP, we observed the effect of repeated application of high pressure on the $[Ca^{2+}]_i$ response. The results of seven

experiments revealed that the response was reduced to 66 ± 13% in the second application of pressure. Thus, the exaggerated $[Ca^{2+}]_i$ responses to hydrostatic pressure in the presence of PTH or CPT-cAMP mentioned above may well be underestimated.

To avoid the blunted effect of pressure, we conducted another series of experiments, in which we observed the effects of 10 nM PTH on $[Ca^{2+}]_i$ in separate tubules perfused with either low or high pressure (0.2 or 1.2 KPa). Although the application of low or high pressure was randomized, the experimental protocol was nonpaired. Representative tracings are shown in Fig. 2. Under the perfusion pressure at 0.2 KPa (Fig. 2*A*), PTH increased $[Ca^{2+}]_i$ only slightly (6 ± 2 nM, n = 12). In marked contrast, when perfusion pressure was at 1.2 KPa (Fig. 2*B*), PTH caused a greater increase in $[Ca^{2+}]_i$ (31 ± 7 nM, n = 5, $P < 0.001$).

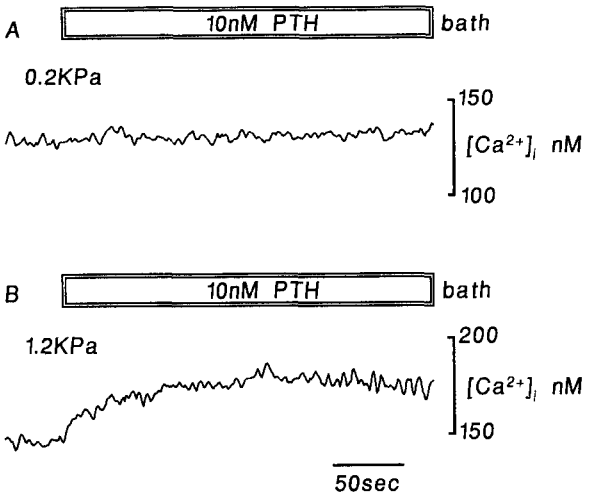

Fig. 2. Representative tracings of $[Ca^{2+}]_i$ in the CNT, showing different responses to PTH in the tubule perfused at 0.2 KPa (*A*) and 1.2 KPa (*B*).

Evidence for Luminal Ca^{2+} Entry

Because Ca^{2+} was omitted from the bathing solution, the basolateral Ca^{2+} entry is not responsible for the pressure-dependent increase of $[Ca^{2+}]_i$. Thus, the source of the increase in $[Ca^{2+}]_i$ is either from the intracellular storage or from the lumen. To examine whether or not the apical Ca^{2+} entry is responsible for the increase in $[Ca^{2+}]_i$, we observed the $[Ca^{2+}]_i$ response to perfusion pressure in the presence or absence of Ca^{2+} in the lumen. The experiments were conducted in the presence of 10 nM PTH in the bath. Figure 3 shows a representative tracing of this protocol. In the presence of 5 mM Ca^{2+} in the lumen, elevation of perfusion pressure caused a sharp increase in $[Ca^{2+}]_i$ as was expected. In marked contrast, in the absence of Ca^{2+} in the lumen, elevation of hydrostatic pressure did not increase $[Ca^{2+}]_i$. When Ca^{2+} was added again in the lumen, an increase in pressure caused a dramatic increase in $[Ca^{2+}]_i$. The similar results were obtained in three experiments. The Ca^{2+} increments in response to pressure ($\Delta[Ca^{2+}]_i$) were 109 ± 9 nM in the control, −4 ± 4 nM in the experimental, and 154 ± 32 nM in the recovery period, respectively. Thus, apical Ca^{2+} entry may be essential for the pressure-dependent $[Ca^{2+}]_i$ increase, although an additional contribution of the Ca^{2+}-induced Ca^{2+} release from the cytoplasmic store cannot be ruled out.

Effect of Nicardipine

Because Bacskai and Friedman (1990) suggested that in the distal straight or convoluted tubules from mouse kidneys PTH increased apical Ca^{2+} entry by incorporating dihydropyridine (DHP)-sensitive Ca^{2+} channels into the apical membrane, we examined the effect of nicardipine on the pressure-dependent increase of $[Ca^{2+}]_i$ in the presence of PTH. As shown in Fig. 4, however, pressure-dependent increase of $[Ca^{2+}]_i$ was not blocked by luminal application of 0.1 μM nicardipine ($\Delta[Ca^{2+}]_i$ = 100 ± 6 nM in control *vs.* $\Delta[Ca^{2+}]_i$ = 144 ± 13 nM in 0.1 μM nicardipine, n = 4). This concentration of the drug is known to be sufficient to block L-type Ca^{2+} channels. To answer the concern that the dose of nicardipine might be too low for the renal tubular effect, we increased the concentration of nicardipine to 10 μM. Even at this high concentration, the pressure-dependent increase of $[Ca^{2+}]_i$ was unaffected ($\Delta[Ca^{2+}]_i$ = 144 ± 13 nM, n = 4).

Effect of CPT-cAMP on Stretch-activated Channels

The $[Ca^{2+}]_i$ measurement experiments described above revealed that a pressure-dependent Ca^{2+} pathway is responsible for the PTH-dependent Ca^{2+} entry via apical membrane of the CNT, suggesting that PTH stimulates a stretch-activated Ca^{2+} permeable channel in the apical membrane. To provide direct evidence in support of this hypothesis, we performed cell-attached patch clamp studies. The everted CNT preparation allowed us an easy access with a patch pipette to the apical (originally luminal) membrane of the tubule. But it made basolateral application of PTH very hard, because PTH must diffuse into a narrow space between the tubule and the inner perfusion pipette. Thus, instead of PTH, we used CPT-cAMP, which showed the same action on the pressure-dependent $[Ca^{2+}]_i$ increase, as an analogue of the second messenger of PTH.

Figure 5*A* illustrates the single channel current recorded from the apical membrane of an everted CNT.

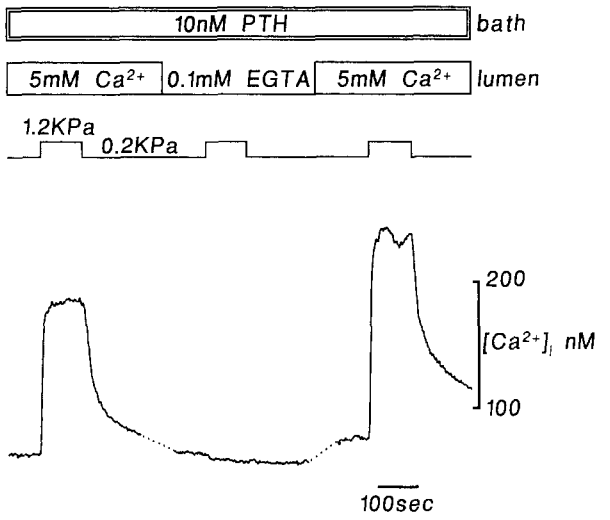

Fig. 3. Representative tracing of $[Ca^{2+}]_i$ showing the effects of removing luminal Ca^{2+} on the pressure-dependent increase of $[Ca^{2+}]_i$ in CNT in the presence of 10 nM PTH in the bath. $CaCl_2$ was omitted from the luminal solution and 0.1 mM EGTA was added during the experimental period. PTH had been applied 5 min before each experiment.

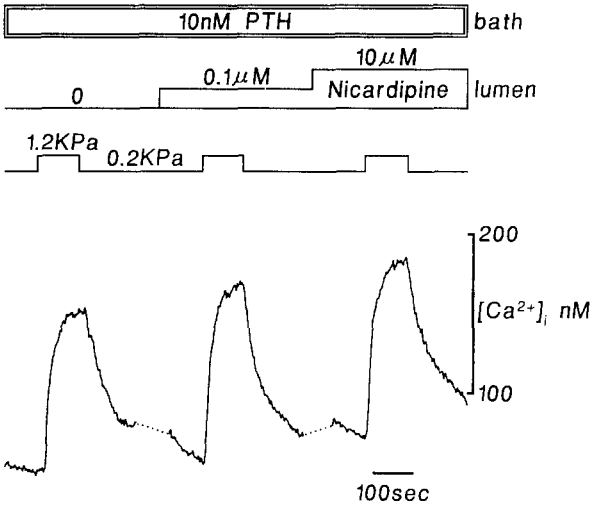

Fig. 4. Absence of effect of nicardipine on the pressure-dependent increase of $[Ca^{2+}]_i$ in CNT superfused with 10 nM PTH. To the luminal solution we added 0.1 or 10 μM nicardipine. PTH had been applied 5 min before each experiment.

Under the control condition, almost no channel opening was recorded from a patch pipette filled with 200 mM $CaCl_2$ ($NP_o = 0.005 \pm 0.001$, $n = 3$) at $V_p = 0$ mV. However, application of negative pressure (−4.9 KPa) in the patch pipette activated inward single channel current ($NP_o = 0.022 \pm .005$, $n = 3$, $P < 0.05$) significantly. Furthermore, addition of 0.1 mM CPT-cAMP to the bath significantly enhanced the stretch-activation ($NP_o = 0.085 \pm 0.014$, $n = 3$, $P < 0.01$) within 1–2 min. The time course of channel activity is shown in the lower panel of Fig. 5*A*.

Similar cooperative actions of membrane stretch and CPT-cAMP were also observed when the patch pipette was filled with 140 mM NaCl, although current size in this experiment (-12.8 ± 0.3 pA, $n = 5$) was much larger than that recorded from the Ca^{2+} pipette (-3.6 ± 0.3 pA, $n = 4$, $P < 0.001$). NP_o was increased from 0.003 ± 0.001 to 0.033 ± 0.001 ($n = 3$, $P < 0.05$) by applying −4.9 KPa negative pressure in the patch pipette, and it was further increased to 0.089 ± 0.024 ($n = 3$, $P < 0.05$) at 1–2 min after addition of 0.1 mM CPT-cAMP to the bath.

Figure 6 shows the results of a representative study in which we examined the effect of CPT-cAMP on the channel. Although we recorded single channel currents for 2–5 min after addition of 0.1 mM CPT-cAMP, NP_o was not significantly changed ($NP_o = 0.004 \pm 0.002$ in control *vs.* $NP_o = 0.002 \pm 0.001$ in CPT-cAMP, $n = 3$). In the final step of every experiment, the stretch-activation of the channel was verified by applying negative pressure in the patch pipette, as shown in this figure. Thus, the stretch-activation is necessary to expect the effect of cAMP.

Current-Voltage Relation and Gating Kinetics

To determine the single channel conductance and the ion species carried by the stretch-activated channel, we analyzed the current-voltage (*I-V*) relation during stretch-activation. In this analysis, we set f_c of the antialiasing filter at 2 KHz to reduce white noise, while f_c was set at 5 KHz to follow a very brief opening event of the channel in the other analysis. We then measured the current size during relatively long openings of the channel. As illustrated in Fig. 7, the inward single channel current was recorded between $V_m - 60$ mV and $V_m + 20$ mV. The slope conductance was 42 ± 2 pS ($n = 4$) obtained from linear regression line ($r = 0.964 \pm 0.007$, $n = 4$). Extrapolated reversal voltage (V_r) was ($V_m + 80.8$) ± 4.3 mV ($n = 4$). We failed to record the outward current from the Ca^{2+} pipette. Because we used 400 mEq Cl^- in the patch pipette, Cl^- current should be outward between $V_m - 60$ mV and $V_m + 20$ mV. Therefore, the single channel current must have been carried by Ca^{2+} in this experiment.

On the other hand, the single channel current recorded from the Na^+ pipette was reversed at ($V_m + 68.5$) ± 1.9 mV ($n = 5$) and quite linear between $V_m - 20$ mV and $V_m + 140$ mV (Fig. 7). The slope conductance obtained from the linear regression line ($r = 0.992 \pm 0.004$, $n = 5$) was 173 ± 7 pS ($n = 5$). In two experiments, we replaced Cl^- with gluconate in the Na^+ pipette. As shown in Fig. 7, the *I-V* relation obtained from the Cl^--free Na^+ pipette was very similar to that from the Na^+ pipette. These results suggest that the current recorded from the Na^+ pipette was carried by Na^+ and K^+.

Figure 8 shows closing kinetics of the stretch-acti-

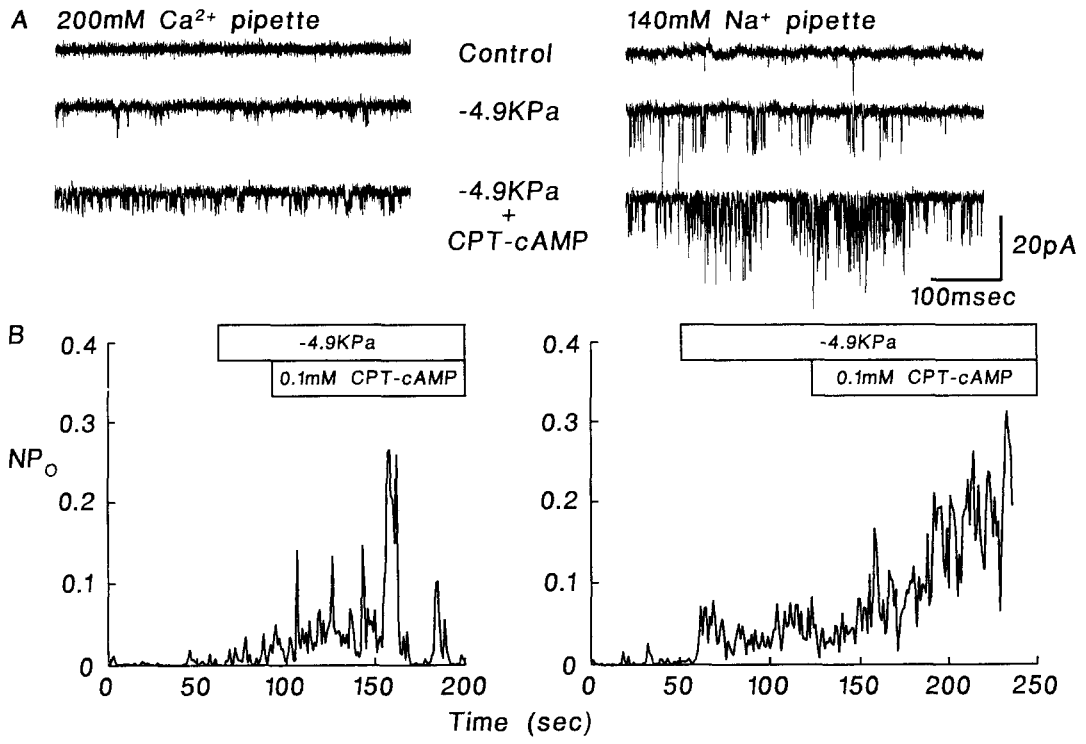

Fig. 5. Effects of membrane stretch and CPT-cAMP on apical (luminal) stretch-activated channel currents in an everted CNT in the cell-attached patch clamp configuration. (*A*) Single channel currents in control, during stretch-activation by −4.9 KPa negative pressure and during stretch-activation plus basolateral superfusion of 0.1 mM CPT-cAMP recorded from patch pipette filled with 200 mM $CaCl_2$ (left panel) and from patch pipette filled with 140 mM NaCl (right panel). (*B*) Time course of NP_o of stretch-activated channel currents recorded from the Ca^{2+} pipette (left panel) and from the Na^+ pipette (right panel). f_c = 5 KHz.

vated channels during membrane stretch. The number of active channels was not clear, even though no multiple channel openings were recorded at the same time. Therefore, we did not analyze the opening kinetics. Histogram analysis revealed two components of closing kinetics with a shorter time constant (τ_1) of 0.15 ± 0.03 msec and a longer time constant (τ_2) of 0.68 ± 0.20 msec in three Ca^{2+} pipette experiments, and with τ_1 = 0.15 ± 0.02 msec and τ_2 = 0.58 ± 0.05 msec in three Na^+ pipette experiments. These time constants in both experiments were not significantly different from each other.

Discussion

Although stretch- or mechanically activated channels have been reported in many types of cells (Morris, 1990; French, 1992), their physiological significance has not been completely understood. However, lines of evidence have accumulated in support of the view that the stretch-activated channels play significant roles in a variety of cell functions. They include cell volume regulation, release of some chemical factors from vascular endothelium, and modulation of smooth muscle contraction, etc. (French, 1992).

Mechanosensitive cation-selective channels have been also reported to exist in the basolateral membrane of renal tubular epithelia (Sackin, 1987, 1989; Ubl, Murer & Kolb, 1988; Hunter, 1990; Kawahara, 1990). It is important to note that all cells handled in these reports were derived from the proximal tubule. Because the proximal tubule is highly permeable to water and large volumetric flux passes through cells, it is reasonable to assume that the mechanosensitive channels play an important role in cell volume regulation. The uniqueness of the present study is that we found a stretch-activated channel in the *apical membrane* of the *distal nephron.* Because the CNT is impermeable to water, cell volume regulation may not be required. Localization of the channel in the apical membrane is also inconsistent with cell volume regulation. Alternatively, we propose that the stretch-activated channel in the apical membrane of the CNT plays an important role in the flow-dependent Ca^{2+} reabsorption.

Properties of Stretch-activated Channels

The stretch-activated channel current recorded from the Na^+ pipette showed linear *I-V* relation without Goldman type rectification (Fig. 6). Thus, this channel could be permeable to all major charge-carrying ions, including Na^+, K^+ and Cl^-, and thus it is nonselective in nature.

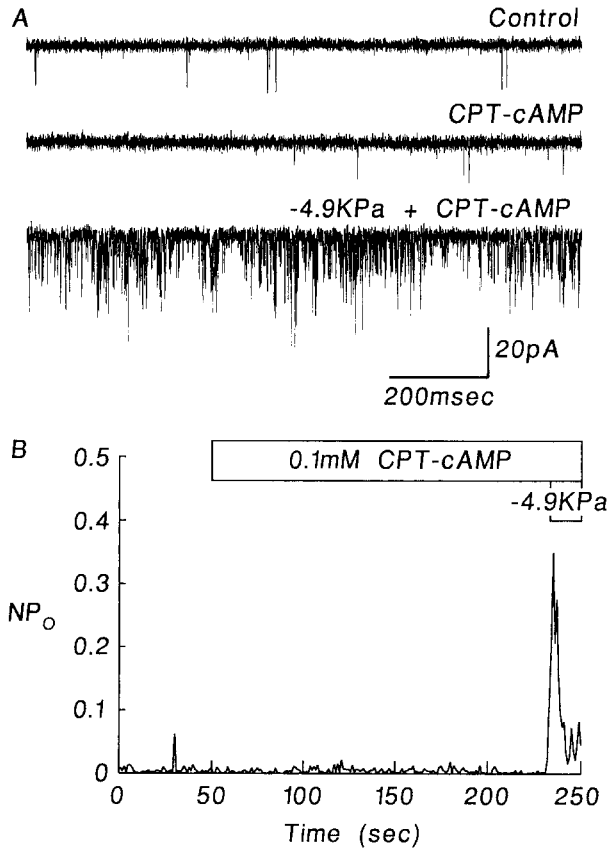

Fig. 6. Absence of effect of CPT-cAMP on channel current without membrane stretch. Single channel current recorded from the Na^+ pipette in control, during basolateral superfusion of 0.1 mM CPT-cAMP and during stretch-activation by −4.9 KPa negative pressure in the presence of CPT-cAMP (*A*). Time course of NP_o is shown in (*B*). f_c = 5 KHz.

However, the contribution of the Cl^- ion as charge carrier would be negligible because there was no difference in the *I-V* relation in the presence or absence of Cl^- in the patch pipette. Because the apparent V_r was $V_m \pm 68.5$ mV and because the V_m in the everted CNT directly measured with the microelectrode was −64.1 mV, the actual V_r of the single channel current evoked by membrane stretch can be estimated to be +4.4 mV in the Na^+ pipette experiments, which is very close to 0 mV. From this V_r and linearity of *I-V* relation (Fig. 7), the ratio of Na^+ and K^+ permeabilities (P_{Na}/P_K) is estimated to be approximately one.[1]

Although we have no direct evidence that the Ca^{2+} ion permeates through the same stretch-activated channel as Na^+ or K^+ ion did, the 42 pS Ca^{2+} permeable channel shares the following common properties with the 173 pS channel: (i) stretch activation, (ii) acceleration of stretch-activation by CPT-cAMP, and (iii) closing kinetics. These common properties strongly suggest that the stretch-activated channel is not only permeable to monovalent cations but also to Ca^{2+}.

To increase the size of Ca^{2+} current through the stretch-activated channel and to minimize Cl^- channel current, we used an extremely high concentration of $CaCl_2$ in the patch pipette. In this experiment, we must take the effect of high ion strength on the channel protein into consideration. However, high ion strength does not seem to affect the observed channel properties including the closing kinetics, because the properties in the Ca^{2+} pipette experiments were very similar to those in the Na^+ pipette experiments, in which normal concentration of NaCl was used for the patch pipette solution. The agreement between closing time constants in both experiments also excludes a noise artifact from the kinetic analysis because signal/noise ratio was much better in the Na^+ pipette experiments than in the Ca^{2+} pipette experiments.

Physiological Roles of Stretch-Activated Channel

Based on the observation that PTH depolarized apical membrane voltage which was unaffected by amiloride, Shimizu et al. (1990*a*) proposed a hypothesis that PTH stimulates nonselective cation channels in the apical membrane through which Ca^{2+} enters in the CNT. The present study provided direct evidence in support of this hypothesis. This is in marked contrast to the Ca^{2+} entry step proposed in the distal straight (CAL) and convoluted tubule (DCT), where Ca^{2+}-selective channels seem to play a more critical role. Bacskai and Friedman (1990) demonstrated that in the cultured renal tubular cells originated from the CAL and DCT of mice, PTH stimulates Ca^{2+} entry from the apical membrane by inserting the Ca^{2+}-selective channel into the membrane. Lau et al. (1991) provided direct patch clamp evidence in support of this view in a similar preparation. By patch clamp on cultured cells from rabbit DCT, Poncet et al. (1992) also demonstrated Ca^{2+} channels in the apical membrane in the presence of PTH. These Ca^{2+} channels are reported to be inhibited by dihydropyridines, although the used doses were very high. We observed that the pressure-dependent Ca^{2+} entry in the CNT was not prevented even by a high dose of nicardipine, indicating that the observed PTH-stimulated Ca^{2+} entry step in the CNT is distinct from the Ca^{2+} channel observed in the distal tubule. Hanaoka et al. (1993) reported that the mechanism of Ca^{2+} exit from the basolateral membrane of the CAL is

[1] Although we have no accurate information on cytoplasmic Na^+ and K^+ concentration, Na^+ (P_{Na}) and K^+ (P_K) permeabilities of the stretch-activated channel may be estimated with Goldman, Hodgkin and Katz (GHK) equations by assuming possible cytoplasmic concentration of Na^+ (15 mEq) and K^+ (135 mEq). From the *I-V* relation described in Fig. 7, P_{Na} and P_K were obtained as 3.7×10^{-13} cm^3/sec and 2.8×10^{-13} cm^3/sec, respectively, by least-squares method. The P_{Na}/P_K ratio could be calculated as 1.3.

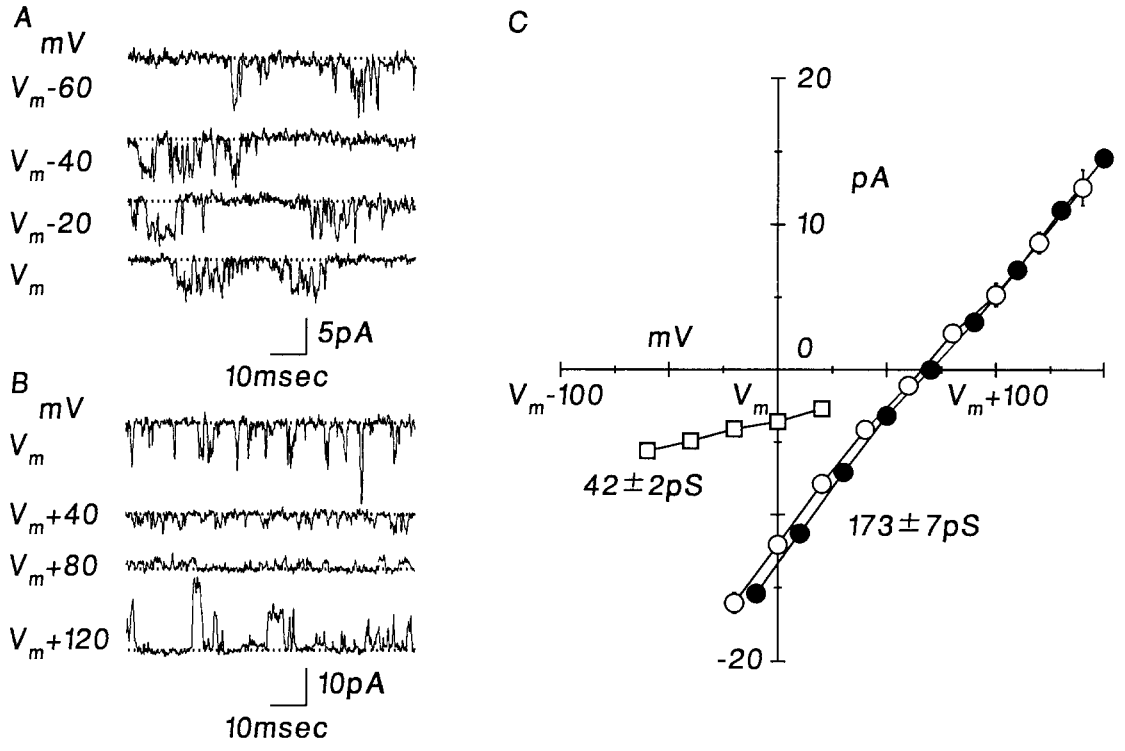

Fig. 7. *I-V* relation of stretch-activated channel current during stretch-activation by applying −4.9 KPa negative pressure in the patch pipette. (*A,B*) Stretch-activated channel currents recorded from the patch pipettes filled with 200 mM $CaCl_2$ (*A*) and with 140 mM NaCl (*B*), respectively, at a variety of membrane voltages. f_c = 2 KHz. (*C*) *I-V* relations of stretch-activated channel currents recorded from the Ca^{2+} pipette (open squares, n = 4), the Na^+ pipette (open circles, n = 5), and the Cl^--free Na^+ pipette filled with 140 mM Na gluconate (filled circles, n = 2).

distinct from that of the CNT: in the CAL, the Ca^{2+} pump plays a critical role, whereas in the CNT the Na^+/Ca^{2+} exchanger is more important. Thus, the mechanisms by which Ca^{2+} is transported across the cell are quite different between the CAL and the CNT. Although the results of the present study indicate that the nonselective cation channel is the major route of Ca^{2+} entry across the apical membrane, we cannot rule out the possibility that there is an additional contribution of the Ca^{2+} channel which is too small to detect under our experimental setting.

Our present study demonstrated that Ca^{2+} transport across the apical membrane of the rabbit CNT was dependent on the luminal perfusion pressure or flow rate. PTH enhanced the Ca^{2+} transport during a higher pressure application, while PTH as well as CPT-cAMP failed to enhance it during lower pressure application. These results are in accord with the findings in the patch clamp experiments that perfusion of CPT-cAMP did not enhance the stretch-activated channel activity without membrane stretch. The contribution of a dihydropyridine-sensitive Ca^{2+} channel can be ruled out as the candidate of the pressure-dependent Ca^{2+} pathway because the pressure-dependent increase in $[Ca^{2+}]_i$ during PTH application was not inhibited by even an excess concentration of nicardipine.

Recently, using the whole-cell patch clamp technique and $[Ca^{2+}]_i$ measurement by Fura-2 in A6 cells derived from frog renal distal tubule, Kawahara and Matsuzaki (1992) demonstrated that flow or shear-stress of ambient solution increased $[Ca^{2+}]_i$ in association with an activation of the Ca^{2+} channel which was not inhibited by 10 μM nicardipine. However, this channel is distinct from the nonselective cation channel found in the present study because the authors showed that Na^+ is impermeable to the channel. Nevertheless, it is interesting to note that the observed phenomenon, i.e., pressure- or flow-dependent Ca^{2+} entry, is very similar. Our unique finding is that this stretch-activated channel is PTH dependent.

K^+ secretion is also known to be flow dependent in the distal nephron including the distal convoluted tubule (DCT), the CNT, and the initial cortical collecting duct (CCD) (Kunau, Webb & Borman, 1974; Good & Wright, 1979). This phenomenon is simply explained by the K^+ concentration gradient across the apical membrane changing as a function of the luminal flow rate. The existence of stretch-activated channels might

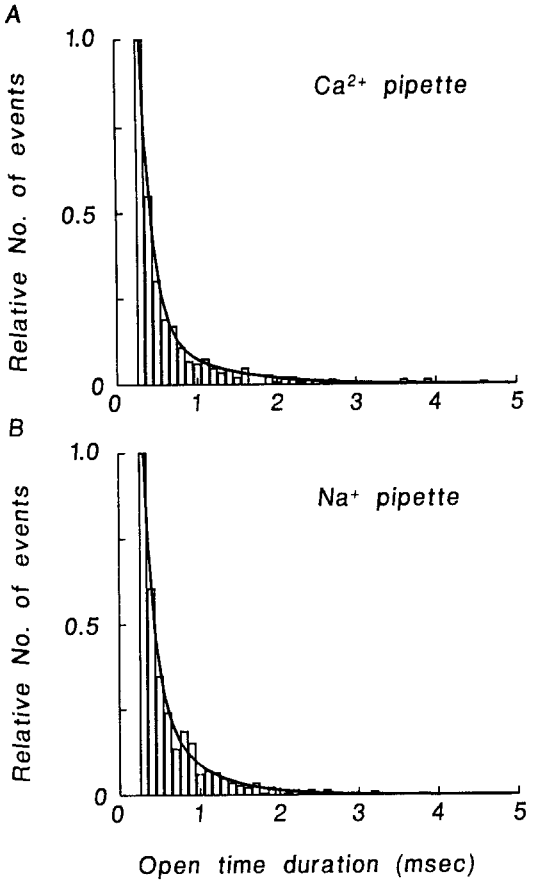

Fig. 8. Closing kinetics of stretch-activated channel during stretch-activation. Frequency histograms of open time durations observed in Ca^{2+} pipette experiments (*A*) and in Na^{+} pipette experiments (*B*). Unbroken curve was plotted by using the following equation: $Y = 4.73 \times \exp(-t/0.14) + 0.22 \times \exp(-t/0.80)$ in *A* and $Y = 4.54 \times \exp(-t/0.14) + 0.44 \times \exp(-t/0.57)$ in *B*, respectively. Both histograms were obtained from 3,200 open events in each experiment. f_c = 5 KHz.

provide an additional explanation for this phenomenon, by assuming the Ca^{2+}-activated K^{+} channels in the apical membrane of the CNT. In fact, Taniguchi and Guggino (1989) suggested the coupling of Ca^{2+} permeable stretch-activated channels and Ca^{2+}-activated K^{+} channels in the apical membrane of the cultured A3 cell line derived from rabbit medullary thick ascending limb. However, more direct evidence is necessary to confirm this notion.

Pressure Dependence or Flow Dependence

It has been reported that shear-stress generated by fluid flow increased $[Ca^{2+}]_i$ in vascular endothelial cells (Mo, Eskin & Schilling, 1991; Schwarz, Droogmans & Nilius, 1992; Davis & Tripathi, 1993) and in A6 cells (Kawahara & Matsuzaki, 1992). In the present study, we perfused the CNT with a leaky collection pipette, so that the actual pressure applied on the apical membrane should be much smaller than the apparent perfusion pressure. Our experimental design, however, could not segregate between the effect of flow and pressure. Under in vivo situations, effects of pressure and flow cannot be segregated. An increase in luminal pressure (0.2 to 1.2 KPa) which increased $[Ca^{2+}]_i$ in our experiments is very close to the luminal pressure measured in the rat distal tubule in vivo, which is rhythmically oscillated between 5.9 mmHg (0.8 KPa) and 8.08 mmHg (1.1 KPa) (Holstein-Rathlou & Marsh, 1989).[2] Thus, the pressure applied in this study was within the physiological range observed in situ, indicating that our finding represents a physiological response to the PTH in increasing Ca^{2+} transport across the apical membrane of rabbit CNT. This view also explains well the discrepancy of the magnitude of Ca^{2+} flux reported by two groups of investigators (Shareghi & Stoner, 1978; Imai, 1981; Shimizu et al., 1990*a*, 1991) because Imai (1981) and Shimizu et al. (1990*a*, 1991) perfused the tubule at higher flow rates, whereas Shareghi and Stoner (1978) did at lower flow rates. Thus, we suggest that the apical stretch-activated channel may be activated by fluid flow or shear-stress, and may play an important role in the flow-dependent Ca^{2+} transport across the CNT.

Conclusion

The Ca^{2+} entry across the apical membrane of the rabbit CNT is flow or pressure dependent. This phenomenon is explained by the existence of a stretch-activated nonselective cation channel which is also permeable to Ca^{2+}. PTH increases Ca^{2+} transport across the CNT by stimulating this channel via the cAMP-mediated second messenger system.

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[2] In the patch clamp study, negative pressure was applied, whereas in the perfusion study positive pressure was applied to the apical membrane. Thus, the directions of the applied pressure were opposite. In the latter case, however, an increase in perfusion pressure caused an increase in the tubular diameter, which in turn may have caused distention of the membrane. Therefore, the stretching of the membrane is the common stimulus in both experimental conditions, although the mechanisms by which membrane distention leads to an activation of the channels are unknown.

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