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Monovalent cation selective channel in the apical membrane of rat inner medullary collecting duct cells in primary culture

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Abstract

Ion channels in the apical membrane of rat inner medullary collecting duct (IMCD) were investigated by the patch clamp technique. Owing to the histological heterogeneity of IMCD, cells were cultured from the lower half of the inner medulla of Wistar rat kidney. Channel activity was rarely seen in cell attached patch, but membrane excision activated multiple units of 28.2 ± 0.7 pS cation selective channel. A Na or K selective channel was not found. The 28 pS channel showed membrane voltage dependency, no rectification, almost equal permeability to monovalent cations ($Na/K/Li/Cs/Rb/NH_4 = 1:1.00:0.82:0.97:1.10:1.71$) and no significant permeation to anions or divalent cations. Calcium of the cytoplasmic side from 10^{-7} M to 10^{-4} M affected the mean number of open channels (nP_o) dose-dependently in excised patch ($IC_{50} = 5 \cdot 10^{-6}$ M). 1 mM of ATP, ADP, AMP and gadolinium reversibly suppressed nP_o to near zero whereas amiloride, cAMP or cGMP had no effect. Multiple conductance substates were frequently observed. These results suggested that this channel belongs to the nonselective cation channels which has been identified in other epithelia and is not responsible for amiloride sensitive Na transport through IMCD cells.

Keywords: Kidney epithelium; Inner medullary collecting duct; Cation channel; ATP; Gadolinium

1. Introduction

Inner medullary collecting duct (IMCD) is the final nephron segment of mammalian kidney. This segment of renal epithelium has been well recognized to absorb water [1] and urea [2] under the control of antidiuretic hormone and to secrete proton [3], thus contributing water and acid base homeostasis. Micropuncture study in vivo [4] showed that IMCD also absorbs Na and secretes K and microperfusion study in vitro [5–9] demonstrated the transcellular Na absorption through IMCD. In cultured IMCD cells, it was observed [10–12] that Na uptake and short circuit currents were inhibited by low dose of amiloride and mineralocorticoid hormone enhanced the short circuit currents. These findings suggest that an amiloride sensitive, Na selective channel exists in the apical membrane of IMCD and plays an important role in Na balance or extracellular volume regulation.

IMCD consists of two heterogeneous subsegments according to histological studies [13–15]. In the upper one third (IMCD1), 90% of the cells are 'principal cells' and 10% are 'intercalated cells' and these cells have the same histological features as those in the outer medullary collecting duct (OMCD). The lower two thirds (IMCD2,3) consist of one type of homogenous cells which have different histological features from IMCD1 and were named as 'IMCD cells' by Madsen et al. [16]. Light et al. [17–19] already reported amiloride sensitive nonselective cation channel in cultured rat IMCD1 cells and suggested its role in Na transport. However, nothing is known about the single channel characteristics in IMCD2,3 which consists of 'IMCD cells'. The present study investigated the property of this channel by applying the patch clamp technique

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to the cells cultured from IMCD2,3. The results showed the presence of a monovalent cation selective channel whose characteristics are different from those of IMCD1 reported by Light and accordingly suggested the functional intrasegmental heterogeneity of IMCD.

A preliminary study was presented at International Symposium on Epithelial Transport (1991 at Okazaki, Japan).

2. Materials and methods

2.1. Primary culture of rat IMCD2,3 cells

8-Week-old male Wistar rat was anesthetized by pentobarbital, both kidneys were removed, the inner medulla of each kidney was carefully cut at its midpoint, and the distal halves were used for the culture. These lower portions of the inner medulla were incubated for 24 h at 4°C in the normal Ringer solution which contained collagenase (234 U/mg *Clostridium histolyticum*, Wako, Tokyo) 1 mg/ml, bovine serum albumin 2 mg/ml, penicillin 100 U/ml and streptomycin 100 μg/ml. Then, softened medulla was minced to 0.1–0.2 mm pieces by sterile small scissors, gently spun down by the centrifugation at 900 rpm for 1 min, supernatant removed, and washed three times by the culture medium containing 5% FBS (fetal bovine serum, Gibco, USA). Washed fragments of IMCD (20–30 pieces) were seeded onto two kinds of support materials, one of which was a small glass slide (10 mm × 10 mm × 0.17 mm) coated by rat tail collagen (Rat tail, Type I, Sigma, USA), and the other of which was a semipermeable membrane CELLGEN (KOKEN, Tokyo) (10 mm × 10 mm × 35 μm, made from bovine atherocollagen [20]). After incubating for 24 h at 37°C in the atmosphere of 5% CO_2, the medium was changed to serum free condition. Culture medium was RPMI1640 (Gibco, USA) which contained L-glutamine 300 mg/l, Hepes 12 mM, HCO_3 23 mM, hydrocortisone 25 nM, aldosterone 1.5 μM, insulin 120 U/l, human transferrin 5 mg/l, sodium selenite 5 μg/l, penicillin 100 U/ml, and streptomycin 100 μg/ml and was replenished every other day.

2.2. Recording of channel current in the patch membrane

Conventional patch clamp technique [21] was applied in this study. Cultured cells were vigorously washed by the bath solution and placed in the small chamber (1 ml volume) which was mounted on the inverted microscope Diaphoto-TMD (Nikon, Tokyo). The 50 GΩ headstage JZ-230J (Nihon Kohden, Tokyo) with the patch pipet was positioned at 45 degrees to the cell surface and its movement was controlled by the micromanipulator MW-4 (Narishige, Tokyo). Patch pipet was made of the hematocrit glass tube (I.D. 1.1 mm O.D. 1.6 mm length 75 mm, Drummond, USA) by modified two stage pipet puller PB-7 (Narishige, Tokyo) and was thickly coated with Silpot 184 (Dow Corning, Japan) from its shank to within 0.1 mm from its tip, which greatly improved its electrical properties. Heat polishing of the pipet tip was done by modified microforge MF-9 (Narishige, Tokyo). When the pipet was filled with 145 mM NaCl, the resistance was from 4 MΩ to 6 MΩ. In the critical experiments which required very low leak current and noise, Corning 7052 or 8161 glass tubes of the same dimension were used. Current signal from the headstage was amplified with the frequency compensation by CEZ-2300 (Nihon Kohden, Tokyo) modified for data acquisition. The final output of channel current from CEZ-2300 was continuously digitized at the sampling rate of 48 kHz with the bandwidth from DC to 22 kHz by 16 bit A/D converter AK-5326 (Asahikasei Microsystem, Tokyo) and stored to the magnetic tape as digital data by modified DTC-57ES (Sony, Tokyo).

Pipet current and bath potential were monitored through an Ag-AgCl wire and an Ag-AgCl pellet, respectively. The pipet potential (V_p) was referenced to the bath and the pipet current (I_p) was defined as positive when the current goes out from the pipet through the patch membrane. Thus, the membrane current by conventional definition is equivalent to $-I_p$ and the membrane voltage is equivalent to $-V_p$ in the case of inside-out patch or to the sum of $-V_p$ and the intracellular potential in the case of cell attached patch. Liquid to liquid junction potential was measured and compensated following the 3 M KCl method [22]. Unless otherwise noted, bath and pipet solutions contained (in mM) NaCl 140, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1, HepesH 5, HepesNa 5, and D-glucose 8.3 and its pH was 7.4 ± 0.05. In the ion selectivity experiment, NaCl of the pipet solution was replaced by an equimolar amount of the desired monovalent cation salt [32]. The calcium sensitivity experiment required the bath solution in which free calcium concentration (from 10^{-7} M to 10^{-5} M) was buffered by 5 mM EGTA [23]. Calcium free bath contained 2 mM EGTA without calcium. Bath exchange was achieved by gentle, simultaneous inflow and outflow of the solution driven by gravitational pressure through two silicon tubes inserted to the bath chamber. All experiments were done at room temperature (22°C).

2.3. Data analysis

Original data stored on the magnetic tape was replayed, converted to analog data, and retrieved to the computer by 12 bit A/D converter GWI-adio (GW instruments, USA) after filtered by 4 pole Bessel filter in modified CEZ-2300. Sampling rate was usually 3600 points/s, sufficiently higher than the filter cut off frequency (−3 dB) of 400 Hz.

As the recorded current showed multiple channel units activity in almost all patch, the mean number of open channel per patch (nP_o) [24] was calculated from I_p at V_p 80 mV and used in the analysis. Open and closed dwell

times in single channel recording were detected automatically by the computer using the half threshold crossing method with cubic spline interpolation [25]. Fitting curves of probability density function were examined by partial (binned) maximum likelihood method [26,27] in both Markov model and fractal model [28]. Likelihood intervals were calculated according to Colquehoun [25]. Data acquisition and its processing were done on Macintosh Operating System (Apple, USA) and the analysis program was developed by the author.

2.4. Statistics

Values were reported as mean ± S.E. Paired or unpaired *t*-test was used in the analysis. Significance was defined as $P < 0.05$.

3. Results

3.1. Cultured IMCD2,3 cells

After seeded in 5% FBS medium, IMCD2,3 fragments adhered to the support material, began to proliferate within a few hours, and spread around the fragments in 24 h. When sufficient amounts of fragments were seeded, cells reached to the confluent state in 5 days in even serum free culture medium. Cells cultured on the collagen coated glass ceased to proliferate after 5 days and began to detach from the glass after 10 days. On the other hand, cells cultured on CELLGEN did not detach even after 3 weeks. They survived much longer and their shape was more globular than those on collagen coated glass.

Fig. 1B shows IMCD2,3 cells cultured on collagen coated glass photographed with a high magnification. Ev-

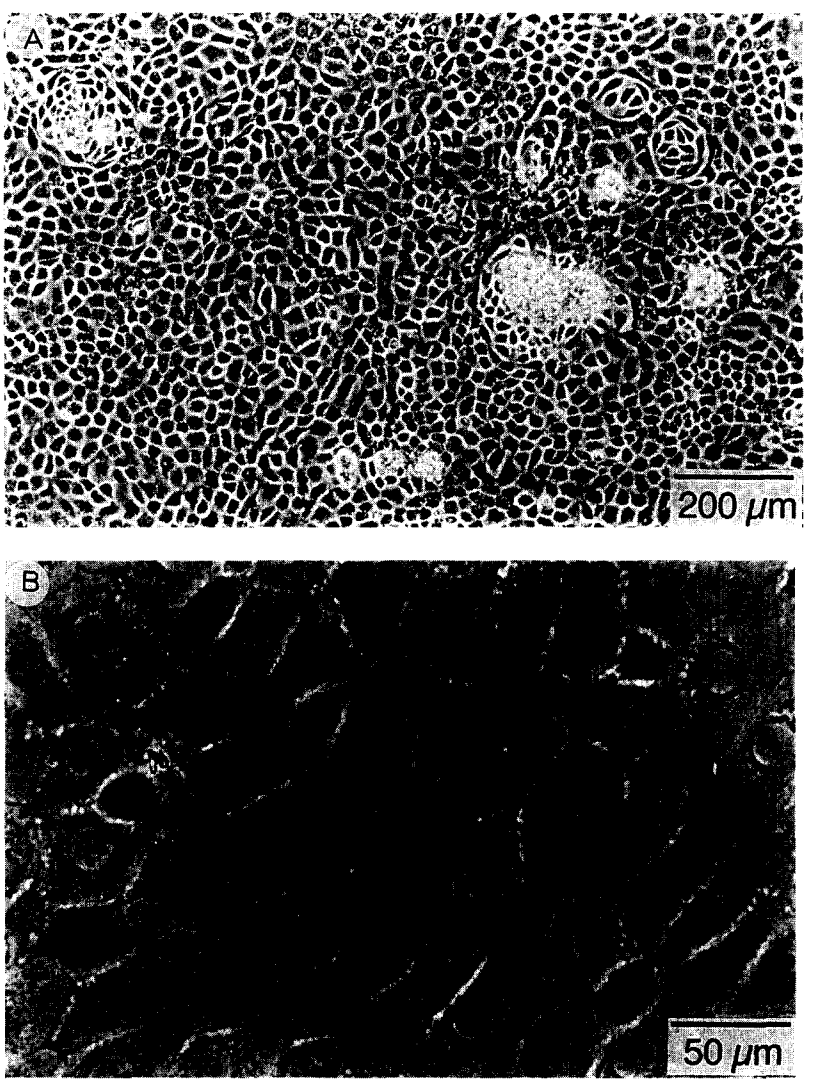


Fig. 1. Phase contrast photomicrograph of 7-day-old cells, cultured on collagen coated glass. (A) Cultured cells proliferated, spread around the IMCD fragments, and were in confluent condition. Small doom formations were seen. (B) Polygonal cells showed almost uniform appearance; fine granules, few organella, large nucleus, and one or two cilia projected in the center. Fibroblasts were not observed.

ery cell showed the uniform appearance with fine granules in the cytoplasma and often one or two cilia in the center, which are compatible with the native IMCD2,3 cell [16]. Occasionally, small doom formation was seen. In the culture condition above, no other kinds of cells were identified. The hypoosmotic shock method which eliminates cells other than IMCD cells [29] was also taken in several cultures but the result was the same. These observations suggested that the cultured cells were homogeneous and consisted of one kind of cell which derived from IMCD2,3.

Most of the experiments used the cells cultured on collagen coated glass of 7 or 8 days old. Cells cultured on CELLGEN of the same amount of days were also examined but the results did not show any difference. So, the data of both experiments were joined and analyzed together. Results described below were extracted from the data of 750 patch recordings. The channel seen most frequently was 25–30 pS size and almost every inside-out patch contained this channel. Large conductance chloride channel (maxi chloride channel) was observed only in less than 1% of the patches. Potassium or sodium selective channel was not found. Therefore, 28 pS channel was analyzed in this study.

3.2. Conductance of patch membrane

With a carefully heat polished pipet, a tight seal between the pipet and the membrane was obtained in a fourth of the cells tried. Although the IMCD cell of native tubule has a thick glycocalyx coat on its apical membrane [15] which might prevent seal formation, a gigaseal could be achieved in the cultured cells without much difficulty. Enzymatic cleaning of the cell surface was not necessary.

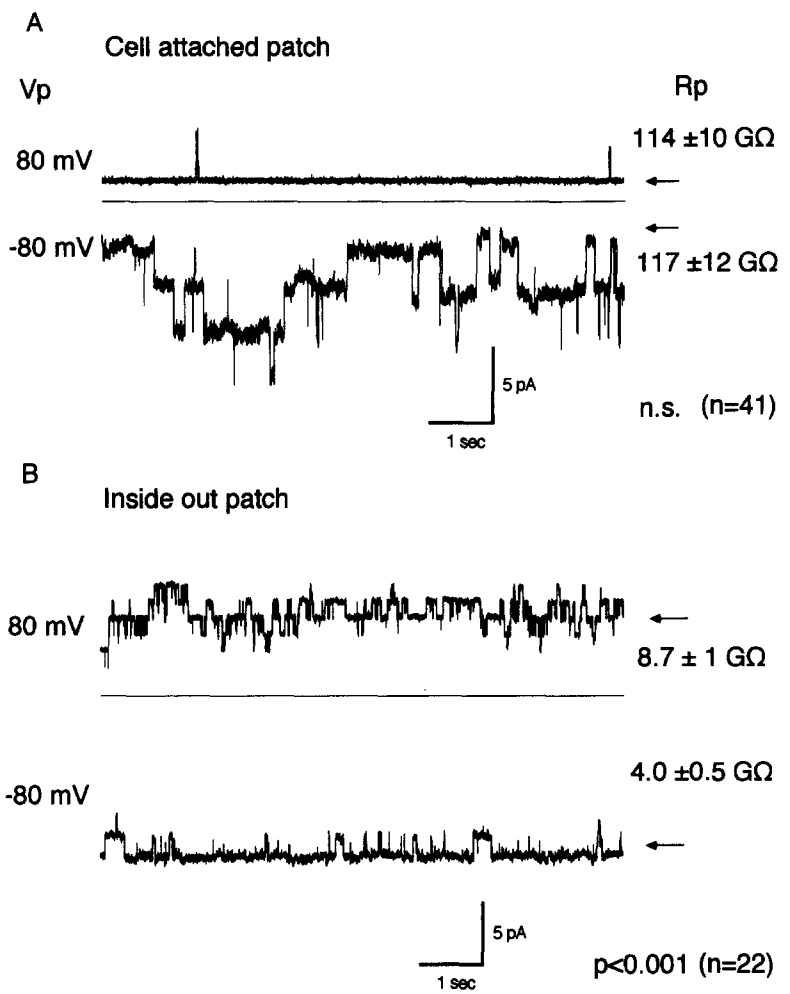


Fig. 2. Resistances of patch membrane (R_p) in cell attached or inside-out configuration. Horizontal lines show the I_p levels at V_p 0 mV. (A) Cell attached patch. The I_p levels of channel closed state at V_p 80 mV and V_p −80 mV were easily identified and indicated by the arrows. R_p values were calculated from these I_p levels, which showed no difference between at V_p 80 mV and at V_p −80 mV. The data of all 41 patches which showed any activity of 28 pS channel were used in the analysis of R_p. (B) Inside-out patch. Because the I_p levels of channels all closed could not be determined for certain, R_p values were calculated from the mean I_p levels which are indicated by arrows. Note the difference of R_p between cell attached patch and inside-out patch, and the marked asymmetry of R_p in inside-out patch. The data of the first 22 patches which showed multiple channel activity from the 713 inside-out patches were used in the analysis of R_p.

For evaluation of the quality of the gigaseal formed between the pipet glass and the cell membrane, the patch membrane resistance (R_p) is calculated by the differences of I_p levels of V_p 0 mV and ±80 mV. Fig. 2 shows these R_p values measured and averaged. In cell attached patch, the channel activity was rarely seen and sufficiently high R_p was observed, which suggested that the R_p approached to the seal resistance itself. In inside-out patch, multiple channel activities were always seen and relatively low R_p was observed, suggesting that the R_p approached to the membrane channel (opened) resistance itself. High R_p values of 114 GΩ at V_p 80 mV and 117 GΩ at −80 mV, were obtained in cell attached patch. On the other hand, much lower R_p values were observed in all inside-out patch membranes. Also, noted was the R_p asymmetry. The R_p of 4.0 GΩ at V_p −80 mV was quite lower than the R_p of 8.7 GΩ at V_p 80 mV in inside-out patch. When inhibitors of the 28 pS channel were applied (shown later as calcium free or ATP or gadolinium experiment), this low R_p of inside-out patch increased or recovered to that of cell attached patch and the asymmetry also disappeared. This indicates that 28 pS channel continues to be open in inside-out patch and its P_o depends on membrane voltage, resulting in low, asymmetrical R_p values. Thus, it is confirmed that sufficiently high resistance seal between the pipet and the apical membrane of IMCD could be achieved in the cell attached patch and maintained through the inside-out patch configuration.

3.3. Cell attached patch

In 750 successfully gigasealed patches, the 28 pS channel activity of cell attached configuration was observed in 41 patches. Of these 41 patches, 39 patches showed very short sporadic opening of the channel which was evoked only by membrane depolarization (negative V_p) and was seen only within few ten seconds after gigaseal formation. After about 5 min, the activity always ceased. Fig. 2A shows one example of high channel activity observed immediately after seal formation. The rest 2 patches continued the channel activities of full unit conductance throughout the experiment ($2/750 < 0.3\%$). The current from one of these 2 patches is shown in Fig. 3. At V_p 0 mV, an inward channel current was seen. The reversal potential was −35 mV in this patch. Assuming that the total concentration of monovalent cations in the cell is almost same as that of the pipet solution, a negative intracellular potential was suggested. The cell potential measured directly by the whole cell clamp varied from −10 mV to −40 mV ($n = 5$, data not shown). There seemed to be no rectification. Its P_o depended on V_p. Negative pressure through the pipette (up to 100 cm H_2O) could not increase the channel activity. Thus, these 28 pS channels seem to remain almost closed in physiological situation and to be insensitive to membrane stretch.

3.4. Activation by membrane excision

Fig. 4A shows an example of the time courses of the channel current being activated by membrane excision. Five experiments were done and four showed the similar current increment as soon as the pipet was removed from the cell membrane. The rest one experiment showed channel activity only after air exposure, suggesting that a vesicle had formed at the pipet tip. Thus, the excision or changing to the inside-out configuration immediately induced multiple current steps of almost same size (28 pS). These current increments clearly demonstrate 28 pS channels being opened by the excision. During the increment of pipet current, no conductance step other than 28 pS size was recognized, suggesting that the conductance difference between the cell attached and the inside-out patch originates entirely from these open channels of 28 pS size. The resistance of the patch membrane decreased from 62.5 GΩ to 1.21 GΩ in this example. These resistances were comparable to the cell attached and inside-out membrane resistance respectively (Fig. 2). The findings above demonstrate that the apical membrane of the IMCD cell has many channels of 28 pS size which remain to be inactive in intact cells and are activated by membrane excision.

Activation mechanisms of channels at membrane excision will consist of several factors which are related to membrane or cytoplasmic substances [30,31]. To examine the role of calcium, membrane excision in calcium free

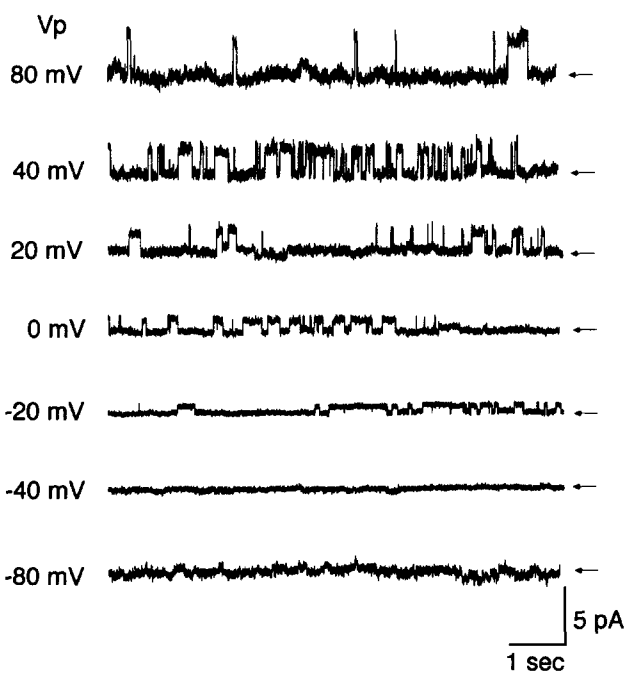


Fig. 3. Current recording in cell attached patch. Only two out of 750 patches showed the consistent channel activity throughout the recordings. Arrows indicate channel closed levels. Inward current was observed at V_p 0 mV. The reversal potential was near −35 mV. There was no rectification. The conductance was 28.8 pS.

condition was done (Fig. 4B). The pipet current did not change at all after membrane excision or air exposure. Five experiments were done and all showed no current increase. This suggested that the condition of high calcium is required in the activation even if channels are released from other inhibitory effects of membrane or cytoplasmic factors. To examine the presence of these inhibitory factors, 20 μM A23187 was applied to the bath (Fig. 4C). Although the intracellular calcium concentration was expected to rise to more than physiological limits by this

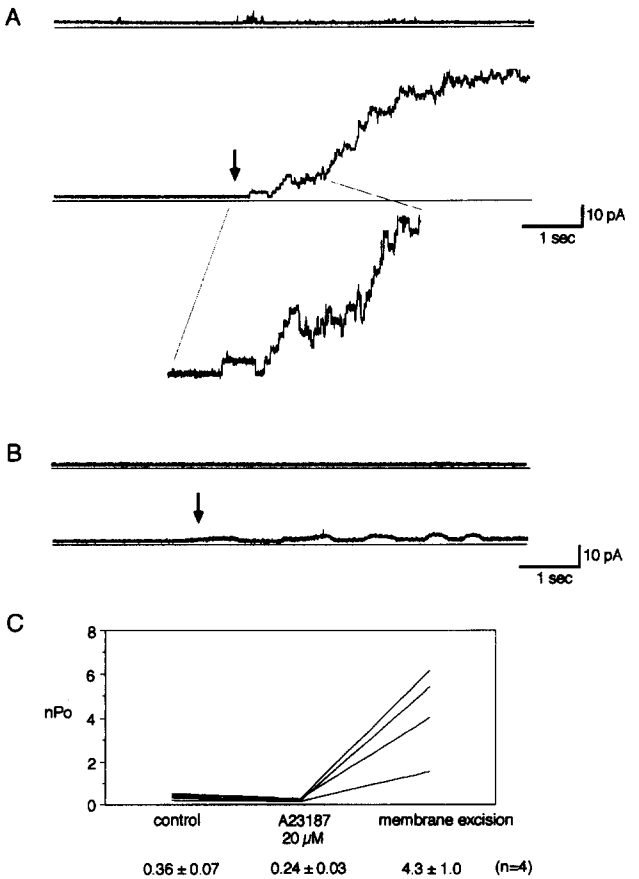


Fig. 4. Time-course of membrane current at excision. Arrows indicate excision points. Horizontal lines show I_p levels at V_p 0 mV. V_p was maintained at 80 mV throughout the experiment. (A) Pipet and bath contained the solution of calcium 1 mM. Inside-out configuration was achieved immediately after moving the pipet off the cell in this example. Multiple steps of unitary current of same size (28 pS) appeared within 2 s after excision. Only very brief channel openings had been observed in cell attached condition. Patch membrane resistances were 62.5 GΩ before excision and 1.21 GΩ after excision. Presented is the representative experiment out of 5. (B) Pipet and bath contained the solution of 0 mM calcium with 2 mM EGTA. The fluctuation of pipet current after excision was due to the capacitance change of the pipet which was moved up the bath solution. The patch membrane resistance did not change and remained at 68.9 GΩ. Presented is the representative experiment out of 5 experiments. (C) The effect of A23187 on nP_o of the cell attached patch membrane. A solution of 20 μM A23187 was applied to the bath. Although the patch contained multiple channels (which was proved by the nP_o increase induced by the membrane excision at the end of experiment), A23187 failed to activate them. The bath solution of A23187 contained Ca 0.15 mM and 20 μM of albumin as a carrier.

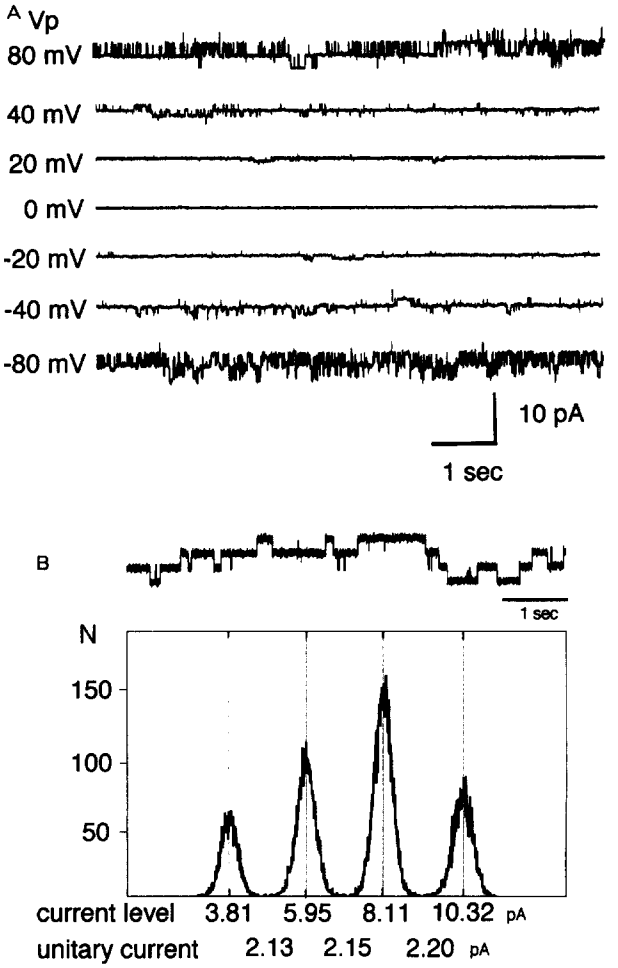


Fig. 5. Inside-out patch recording. (A) Almost all patches showed multiple channel activities. At positive V_p, the channel current reached full unit amplitude and stayed long enough to enable an easy measurement of the unit current. At negative V_p, the channels tended to be open and fast open/close events were prominent, often resulting in the fluctuation or drift of the current level. The same phenomenon was observed in cell attached patch (Fig. 3). No rectification was observed. (B) Histogram of current amplitude at V_p 80 mV. Almost equal units of channel current and binomial distribution curve were identified. This recording did not contain subconductances.

calcium ionophore, no current increase was observed. This suggests that high calcium alone is not sufficient to open the channels and some other inhibitory factors of intact membrane or cytoplasmic substances exist which can keep the channel inactive even in high calcium condition. In fact, cytoplasmic ATP, ADP have strong inhibitory effects on this channel (Fig. 10) and may partly contribute to the inhibition.

3.5. Inside-out patch

In inside-out configuration, almost all patch (713/750 > 95%) contained multiple channels which suggested the high density or the clustering of this channel in the apical membrane. Fig. 5A shows a typical channel current. At

positive V_p, clear openings to full amplitude were usually seen whereas flickerings were always observed at negative V_p. There was no rectification. Fig. 5B shows the histogram of current amplitude in which three units of channel current were observed. Its unitary conductance was 28.2 ± 0.7 pS ($n = 6$) in symmetrical 145 mM NaCl solution, which was not different from cell attached patch.

The calcium sensitivity suggested above was further examined in inside-out patch. When calcium free solution with 2 mM EGTA was applied to the cytoplasmic side of patch membrane, no unit current of 28 pS channel was observed and the mean current level decreased dramatically to that of cell attached patch and it partially recovered after 1 mM Ca was applied (Fig. 6A and B). This means that the activity of the channel was completely suppressed by the Ca free solution of the cytoplasmic side. In calcium free solution, much smaller, channel openings

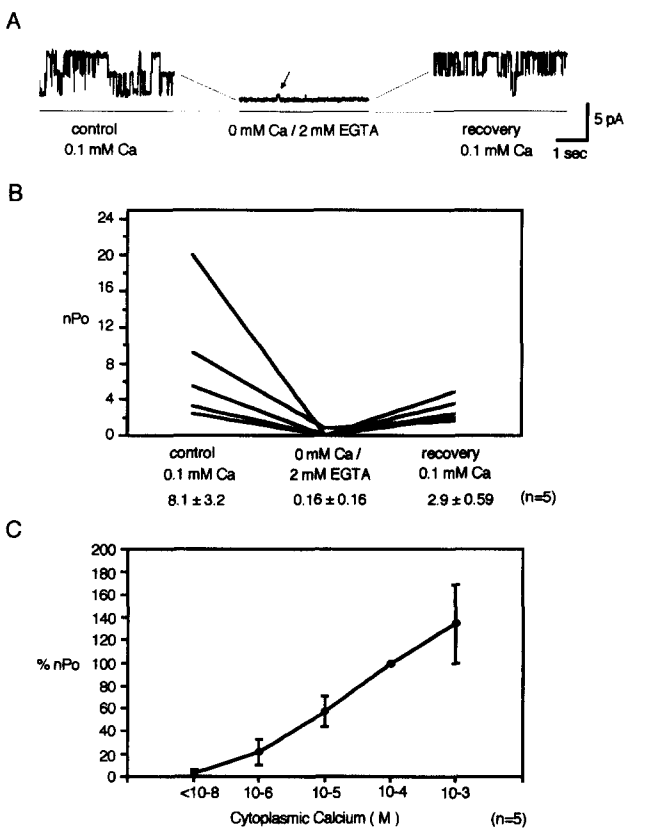


Fig. 6. Sensitivity to calcium of cytoplasmic side. (A) Bath solution was exchanged to Ca free solution in inside-out patch. Control and recovery periods had Ca 10^{-4} M bath and experiment period had Ca free bath. V_p 80 mV. The channel activity was completely inhibited. No full opening of the 28 pS channel was observed. The much smaller unit current which was indicated by the arrow was occasionally seen. The patch membrane resistance increased from 11.3 GΩ to 44.5 GΩ. (B) Inhibition of nP_o by Ca free solution. Five experiments (same as in A) were done and the change of nP_o was shown. (C) Dose-response curve of $\%nP_o$ to calcium. The same protocols as in B were done. Ca in the control period was 0.1 mM and Ca in the experiment period was from 10^{-7} to 10^{-3} M. Inhibition ratio ($\%nP_o$) was defined as the ratio of nP_o at experiment to nP_o at control. Five patch membranes were investigated in each concentration of Ca. IC_{50} was 5 μM.

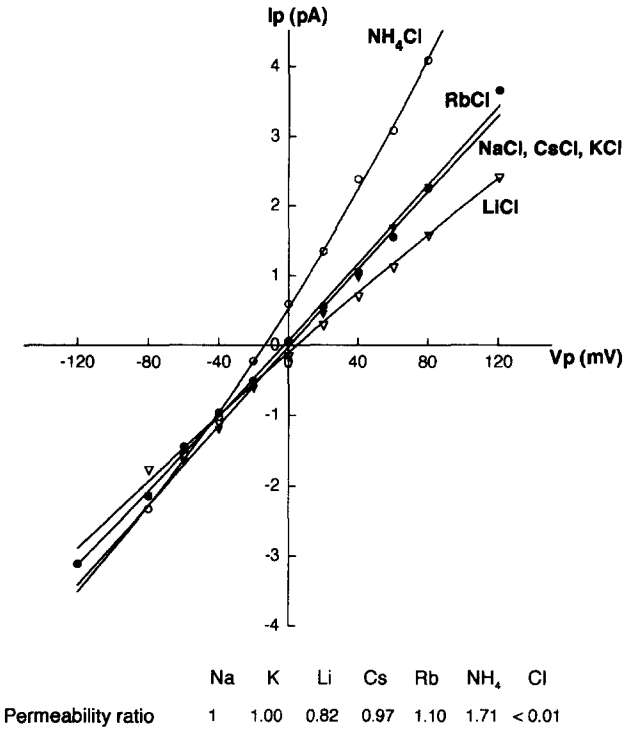


Fig. 7. I_p / V_p plot and ion permeability of the 28 pS channel. Bath contained 145 mM NaCl. Pipet contained various monovalent cations of 145 mM. The plot was fitted by the Goldman-Hodgkin-Katz current equation using least-squares method. The permeability of each ion was obtained as the best fit permeability parameter of the equation and its ratio to P_{Na} was shown. No rectification was observed in the range of V_p from −80 to 80 mV, when the membrane faced symmetrical NaCl 145 mM solution.

or flickerings were seen occasionally, which might be subconductance states or unresolvably fast open/close states of 28 pS channel. It is difficult to answer whether these small currents indicate other different channels or subconductances of 28 pS channel, but the authors believe that these are the latter because these currents shared almost same characteristics as 28 pS channel (see following results of subconductance). Fig. 6C demonstrates that this channel is sensitive to the cytoplasmic calcium of broad range concentration from 10^{-7} M to 10^{-3} M. IC_{50} was around $5 \cdot 10^{-6}$ M. The nP_o of recovery periods was lower than that of control periods. Because long observation of inside-out patch recordings showed very gradual decrease of nP_o, this incomplete reversibility might be partly explained by the time-dependent decrease of channel activity after membrane excision.

3.6. Ion selectivity

The permeability of various ions was estimated from the fitting of Goldman-Hodgkin-Katz current equation in inside-out patch (Fig. 7). The channel showed almost equal permeability to various monovalent cations. Ammonium ion had considerably higher permeability than the other cations. Permeability of Na was $5.06 \cdot 10^{-14}$ cm^3/s.

Sodium gluconate in the pipet shifted the reversal potential (V_{rev}) only to less than 2.0 mV and reducing NaCl to 70 mM in the pipet shifted V_{rev} up to 20 mV, suggesting that chloride ion does not permeate significantly. Fitting the G-H-K equation resulted in P_{Cl}/P_{Na} ratio less than 0.01. Divalent cations such as Ca and Mg also showed no significant permeability. Because the cell attached experiments of KCl in the pipet showed the same I_p/V_p curve as inside-out patch, equal permeability of K ion to Na ion was suggested also in the intact cell condition. These results show that this channel is not Na selective but monovalent cation selective.

3.7. Membrane voltage dependency

As suggested in previous results, the nP_o of this channel was clearly dependent on the membrane voltage in both cell attached and inside-out patches as shown in Fig. 8A and B. In every patch, depolarization of the patch membrane increased nP_o and hyperpolarization decreased it. This sensitivity to membrane voltage was further confirmed by outside-out patch configuration ($n = 3$, data not shown), which denied the possibility that the voltage dependency may come from some soluble impurity of the pipet glass which blocks the channel. The response of nP_o to V_p alteration was not so fast and usually took more than 1 s (Fig. 8C). In cell attached patch, hyperpolarization decreased nP_o to minimum level, less than 0.01. Considering the physiological condition of membrane voltage, this V_p dependency again indicates that almost all channels are closed in intact cells.

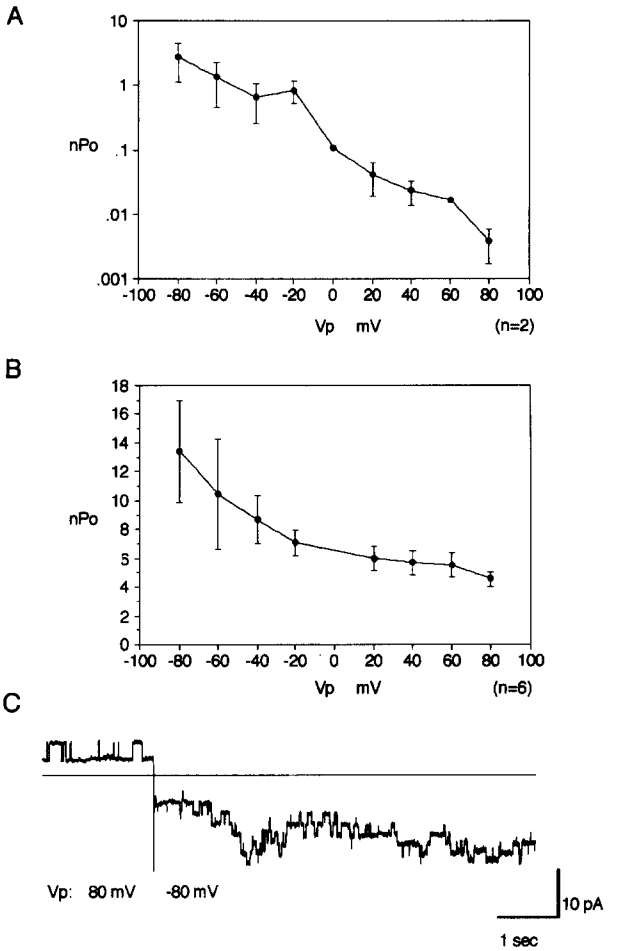


Fig. 8. Membrane voltage dependency. (A) Cell attached patch. (B) Inside-out patch. Relation between nP_o and V_p is shown. Sequence of changing V_p was randomised, repeated more than three times, and the nP_o during the each V_p holding time (8 s) was averaged. nP_o obtained in this fashion at each V_p from different patch was averaged and reported. Numbers of patch membrane are shown as n. Vertical bar shows S.E. Hyperpolarization of the membrane suppressed nP_o. nP_o at V_p 80 mV was significantly different from that at V_p −80 mV in both A and B. (C) V_p was changed abruptly from 80 to −80 mV during the recording of inside-out patch. Horizontal lines indicate current level at V_p 0 mV. Closed channels were opened within 1 s after V_p was changed to −80 mV.

3.8. Insensitivity to amiloride

It is already proved that low dose of amiloride effectively inhibits Na transport through the IMCD2,3 cells by microperfusion study [5]. Whether this 28 pS channel has amiloride sensitivity is an important issue. Fig. 9 demonstrates that amiloride which was applied from either cytoplasmic or luminal side could not block this channel activity in inside-out patch membrane. With the pipet filled with 10 μM amiloride, nP_o did not show a significant decrease compared with the control group of no amiloride in the pipet (Fig. 9A1). Also bath exchange to amiloride solution did not produce significant difference (Fig. 9B1). As amiloride has positive charge in pH 7.4 solution and its blocking effect will be expected to be dependent on V_p, voltage dependency of blocking effect was also examined. The voltage dependency ratio (nP_o at V_p 80 mV divided by nP_o at V_p −80 mV) was not different from the control value (Fig. 9A2 and B2). High concentration of amiloride (100 μM) is known to completely inhibit the Na current through the epithelial Na channels [33]. Under the condition of 100 μM amiloride in the pipet, apparent multiple 28 pS channel activities with no flickering at V_p 80 mV were still observed ($n = 3$, data not shown). These results suggest that the 28 pS channel is insensitive to the amiloride which is a specific inhibitor to the epithelial sodium channel, and accordingly seems not to be responsible for Na transport through IMCD2,3 cells.

3.9. Response to other compounds

As shown above, this channel shares quite common features of nonselective cation channels (NSCCs) found in various tissues. So, other features of these channels were examined and confirmed. Fig. 10 clearly shows the inhibitory effect of 1 mM gadolinium and adenosine phosphates. They reversibly suppressed nP_o to near zero. The effect of cGMP or cAMP was not significant. ATP of 1

mM seems to be near the physiological level in the cell and may be one of the inhibitory factors in the intact IMCD cells.

3.10. Multiple levels of subconductance

During these experiments, frequently observed were the unit currents much smaller than 28 pS. Fig. 11 shows the typical patterns. One cannot deny the possibility that these small unit currents represent an other new channel. However, these small currents appeared also in the condition of pipette filled with K, Li, Rb, Cs, and NH_4. They showed the same V_p dependency as 28 pS channel and were not affected by 10 μM amiloride. They were also observed at positive V_p in the experiment of bath sodium gluconate and of divalent cation free pipet solution. These features were similar to 28 pS channel and suggested that they were not the other new small channels, but most likely the subconductances of 28 pS channel [34]. At least, two substates of half open (19 pS) and half closed (10 pS) were recognized. The conditions or factors which induce subconductances are not clear and to be determined.

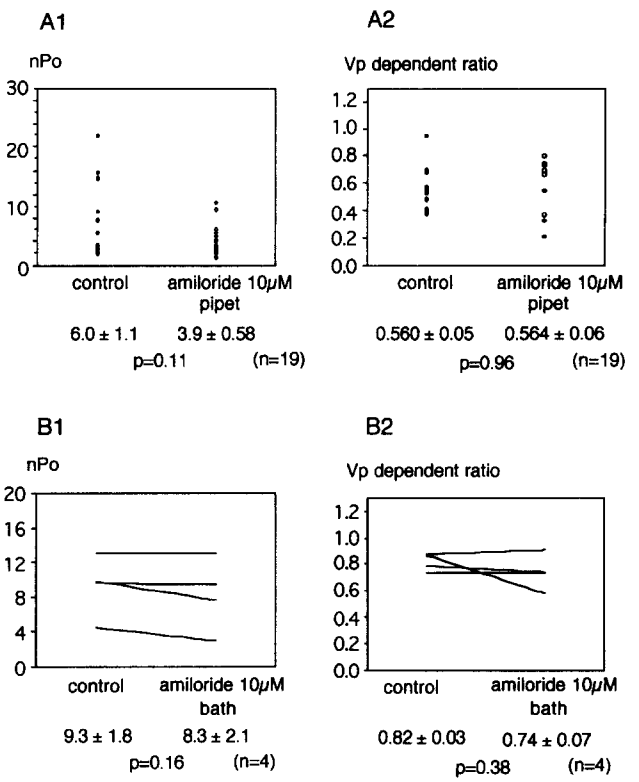
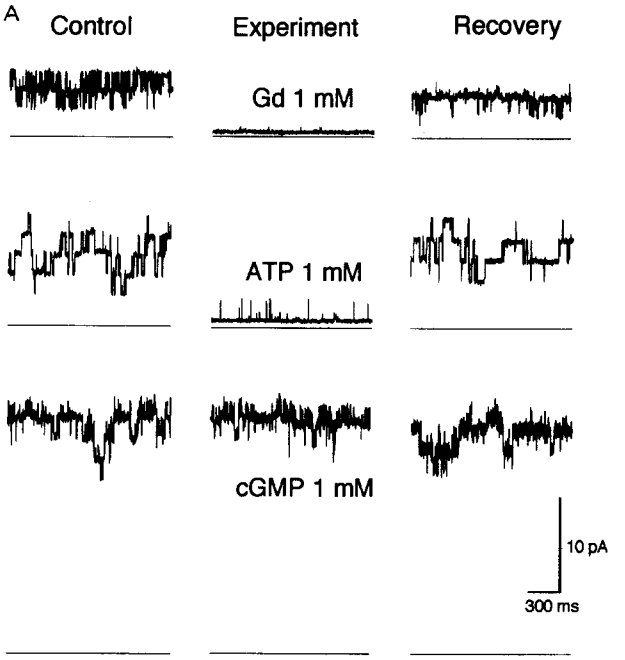


Fig. 9. Amiloride effect on nP_o of inside-out patch. V_p 80 mV. A1: Amiloride was applied to luminal side (pipet solution). The control group contained no amiloride and the experiment group contained 10 μM amiloride in the pipet solution. Control and experiment used different patch membranes. A2: Voltage dependency ratios (nP_o at 80 mV divided by nP_o at −80 mV) were calculated and plotted from the data of control and experiment groups of A1. B1: Amiloride applied to cytoplasmic side (bath solution) of the same patch membrane. Bath was exchanged to 10 μM amiloride solution. B2: Voltage dependency ratios calculated from B1 were shown. Unpaired analysis in A1 and A2 showed no difference. Paired analysis in B1 and B2 showed no difference.



B

	n	control	experiment	recovery
Gadolinium	3	3.1 ± 0.3	*0.37 ± 0.15	1.8 ± 0.1
ATP	4	3.5 ± 0.7	*0.85 ± 0.19	3.7 ± 0.4
ADP	4	3.8 ± 1.0	*0.62 ± 0.09	2.7 ± 0.7
AMP	4	4.5 ± 0.8	*0.77 ± 0.15	3.0 ± 0.7
cGMP	5	10.7 ± 2.4	9.9 ± 2.0	9.5 ± 1.6
cAMP	5	7.2 ± 2.2	6.7 ± 1.9	8.2 ± 2.0

Fig. 10. Effects of ATP, gadolinium, and cGMP on nP_o of inside-out patch. V_p 80 mV. (A) Examples of channel current recordings in control, experiment, and recovery are presented. 1 mM of gadolinium, ATP, cGMP was applied to cytoplasmic side (bath solution). Horizontal line shows the current level at V_p 0 mV. Gadolinium and ATP increased the membrane resistance from 16 GΩ to 95 GΩ and from 10 GΩ to 72 GΩ, respectively. cGMP did not inhibit nP_o significantly. (B) Responses of nP_o to various substances were shown. Asterisks indicate significant difference of $P < 0.05$.

3.11. Distribution of open and closed dwell time

A few recordings of inside-out patch contained the single channel current suitable for this analysis. Fig. 12 shows one example of the dwell time distribution. Although the stability plot [27] (not shown) indicated no modal transition, at least three exponentials in the Markov model were required in fitting both open and closed dwell time distribution. The fractal model using two variables fitted quite well. Several studies reported that the fractal model fits dwell time distribution of NSCCs as well as, or better than the Markov model [28,35]. This may suggest the possibility that the fractal behavior is a specific feature of the NSCCs. However, it is difficult to say which model

is better in describing this 28 pS channel behavior due to the limited number of dwell times and recordings. The results suggested at least that this channel has multiple (more than three) conformations of open or closed states and complex kinetics.

4. Discussion

The present study demonstrates that a monovalent cation selective channel exists in the apical membrane of IMCD2,3 cells. This channel shows (1) selectivity to monovalent cations, no permeability to divalent cations or anions; (2) full unitary conductance of 28 pS, subconductances seen frequently; (3) activation by membrane excision; (4) membrane voltage dependency; (5) sensitivity to calcium of cytoplasmic side; (6) high density; (7) no activation by membrane stretch; (8) insensitivity to amiloride; (9) inhibition by gadolinium, ATP, ADP, AMP; and (10) insensitivity to cAMP, cGMP. These characteristics of the channel are partially different from those of the nonselective cation channel (NSCC) in cultured rat IMCD1 cells reported by Light et al. [17]. Their results are that the channel is sensitive to submicromolar amiloride and is inhibited by cGMP, and its P_o is independent of membrane voltage. As other features such as the sensitivities to gadolinium, ATP, and calcium are not investigated in their study, it cannot be concluded that these two channels are completely different entities. The reason of the different results is not clear, but two possibilities can be mentioned. First, the intrasegmental heterogeneity may explain the differences. Light et al. used the cells which were cultured from outer two thirds of rat IMCD. This means their cells may derive from IMCD1 whereas the cells in this study derive from IMCD2,3. Principal cells of IMCD1 distinctively differ from IMCD cells of IMCD2,3 at histological point as mentioned in the Introduction. Functional intrasegmental heterogeneity of IMCD which matches this histological heterogeneity has been also demonstrated about urea and water permeability and its response to vasopressin [36]. So, the differences of channel characteristics or behaviors between IMCD1 and IMCD2,3 appear to coincide with this histological heterogeneity in IMCD, suggesting that IMCD2,3 has the channel protein different from that of principal cells in IMCD1 or at least different mechanism of channel modification. Second, the

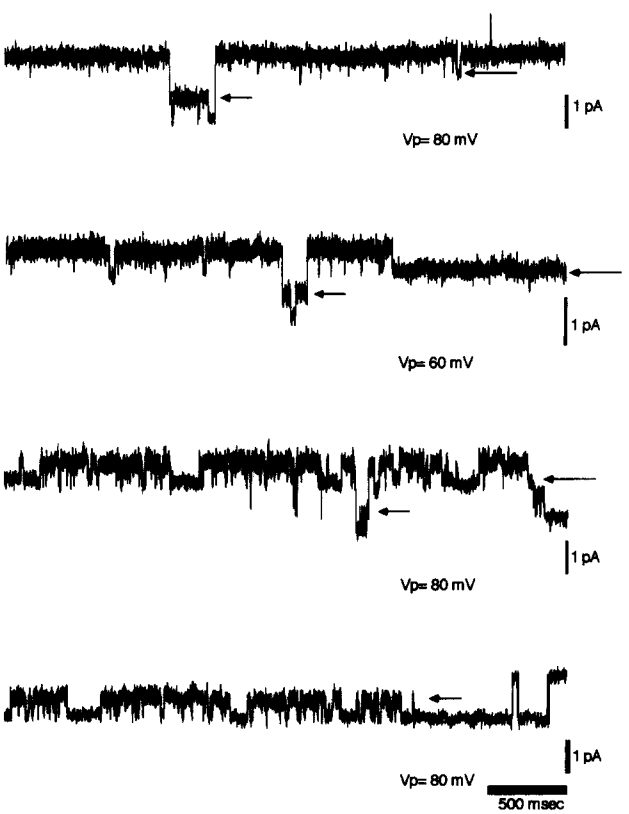


Fig. 11. Subconductances of 28 pS channel. Channel recordings of inside-out patch were shown. Long arrows indicate half open (19 pS) and short arrows indicate half closed (10 pS) states.

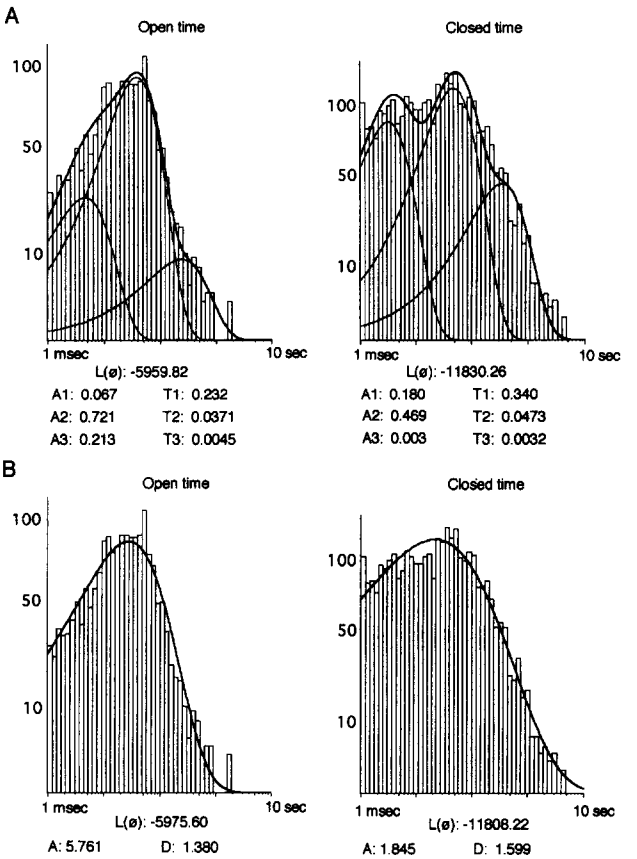


Fig. 12. Sigworth-Sine plot of the channel dwell time. Abscissa is the log binning of dwell time. Ordinate is the square root of number of events observed in each bin. Curves were fitted by the partial maximum likelihood method (50 bins from 1 ms to 10 s). Logarithm of likelihood was shown as $L(\phi)$. Channel current was filtered at 1600 Hz and digitized at 14400 points/s. (A) Markov model fitting. Three time constants (in unit of second) were required for better likelihood than the fractal model. (B) Fractal model fitting. A and D represents fractal set point (in unit of s^{-2}) and fractal dimension, respectively.

culture condition may influence the expression of channels. For example, in A6 cell line, various channels including small Na selective channels and NSCCs have been observed [24,37,38] and the dominant channel differed depending on the culture conditions. It is undeniable that subtle difference in the culture condition of these IMCD cells may result in the different characteristics of the same channel.

This channel shares many features with NSCCs which have been reported in other tissues including epithelial cells. In cultured cell or cell line, mouse fibroblast L cell line [39], Ehrlich ascites tumor cell [40], mandibular gland cell [41], pulmonary alveolar cell in primary culture [42], and A6 cell line [38] are reported to have NSCCs. Native tissues such as pancreatic acinar cell [43], marginal cell of cochlea [44], distal colon cell [45], cerebral capillary endothelial cell [46], and vestibular dark cell of semicircular canal [47], have the same kind of NSCCs. Accordingly, the NSCCs are not peculiar to cultured or undifferentiated cells but do exist in the well differentiated native cells. In renal epithelium, cortical collecting duct cells in primary culture [48,49] and mouse cortical collecting duct [50] have NSCCs. The NSCCs observed above are, in common, 20–35 pS size, almost closed under physiological conditions, activated by membrane excision, are often sensitive to calcium, ATP, gadolinium, and exist in a cluster. Usually, they are insensitive to amiloride and their physiological role is not yet clearly determined. These NSCCs are not identical because some features differ from channel to channel, suggesting the diversity of the NSCC group. It is possible that the minor differences of channel characteristics come from the posttranslational modification specific to each tissue.

The high density of this channel in the apical membrane of IMCD cells should be noted and may suggest the possibility of its physiological roles. If it is activated even at very low P_o, a significant amount of Na absorption or K secretion or NH_4 transport will occur depending on the cation concentration of luminal fluid (urine). If the inflow or outflow of these cations accompanies an equivalent amount of anion, cell volume changes will occur. In other tissues, activation of NSCC in cell swelling has been reported [40]. As the renal medulla is affected by the osmolality change, this channel may also play a role in cell volume regulation.

The Present study could not find Na selective channels which are highly sensitive to amiloride and contribute to transcellular Na absorption in IMCD2,3 [5]. In other nephron segments, Na selective channels have been identified. Cortical collecting duct has amiloride sensitive Na channels [49]. A6 cells have a small Na selective channel which is sensitive to amiloride, induced by aldosterone and responds to vasopressin [24]. It is shown in cultured cortical collecting duct cells [48] that amiloride induced asymmetrical I_p of patch membrane which suggested the presence of amiloride sensitive, unresolvably small Na channels. It can not be denied that the apical membrane of IMCD2,3 also has Na selective, amiloride sensitive channels which are too small or have too fast kinetics to be detected by our recording system. In such case, fluctuation analysis of whole cell or patch membrane current will be able to detect the amiloride sensitive small channel and its characteristics, but has not yet been reported.

Lewis et al. [51,52] described that Na channel in rabbit urinary bladder epithelium changes its characteristics through the degradation by proteinases. It loses Na selectivity and amiloride sensitivity and changes its unit conductance. It is finally washed away from the epithelium by subsequent degradation. It is possible that the channel of the present study is a degraded Na channel by urinary serine proteinase such as kallikrein. On the other hand, however, activation of the NSCCs by physiological substance has also been reported. PGE_2 activated NSCCs in rat colon crypt cells [53]. The NSCC in pancreatic acinar cell was activated by cholecystokinin in physiological level [54]. Platelet derived growth factor activated NSCC in mouse fibroblast [39]. Thus, it remains to be clarified whether the 28 pS monovalent cation selective channel in the apical membrane of IMCD2,3 cell is already the degraded channel waiting to be removed off, or rather the matured, pre-stimulated channel waiting to be activated by physiological substances.

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