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Characterization of Two Mammalian Cortical Collecting Duct Cell Lines with Hopping Probe Ion Conductance Microscopy

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Abstract We morphologically and physiologically characterized Madin–Darby canine kidney (MDCK) cell and mouse principal cell of kidney in cortical collecting duct (mpkCCD) via hopping probe ion conductance microscopy, transepithelial electrical resistance (TEER) measurements, and single-channel recordings. The specific membrane structures of microvilli and tight junctions were clearly observed in MDCK and mpkCCD cell monolayers. The electrophysiological functions of epithelial Na^+ channel in MDCK and mpkCCD cells were further characterized by measuring amiloride-sensitive TEER values for the whole-cell monolayer and detecting the ion channel activities with patch clamping. Our results provide more morphological and functional information to help better utilize these two mammalian CCD cell lines for mechanism studies of sodium absorption and reabsorption in the distal nephron.

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Introduction

Renal cortical collecting duct (CCD) epithelia play a key role in Na^+ absorption and reabsorption, as well as extracellular volume regulation (Schild 2010; Grossmann and Gekle 2009; Pratt 2005). Epithelial Na^+ channels (ENaCs), which are expressed in the apical membrane of CCD, are the rate-limiting regulators for Na^+ absorption and reabsorption in the distal nephron (Dooley et al. 2012; Staruschenko et al. 2007). We previously used scanning ion conductance microscopy to demonstrate that *Xenopus* renal epithelial A6 cells were a good CCD cell model to investigate the regulation of ENaC (Zhang et al. 2005, 2007; Gorelik et al. 2004; Gorelik 2005). However, the amphibian original of the A6 cell line may be different from a mammalian CCD cell (Shane et al. 2006).

In recent years, researchers have established some mammalian CCD cell lines, such as M1, Madin–Darby canine kidney (MDCK), and mouse principal cell of kidney in cortical collecting duct (mpkCCD) (Korbmacher and Barnstable 1993; Takemoto et al. 1992; Summa et al. 2001). Among these, MDCK and mpkCCD cells are two widely used mammalian CCD cell models (Ishikawa et al. 1998; Kortenoeven et al. 2011; Karpushev et al. 2010; Nascimento et al. 2007). However, morphological and functional assessments of these two mammalian CCD cells are still lacking. The morphology information of fixed MDCK cells was previously obtained by scanning electron microscopy (SEM) and atomic force microscopy (AFM) (Lesniewska et al. 1998; Nascimento et al. 2007). Compared with AFM and SEM, noncontact hopping probe ion conductance microscopy (HPICM) is more suitable for

imaging living cells in their physiological culture condition at high resolution (Novak et al. 2009; Rheinlaender et al. 2011). In this study, a newly developed HPICM was used to assess the morphology of living MDCK and mpkCCD cells; then the electrophysiological functions of both cell lines were further characterized with patch clamp recording and transepithelial electrical resistance (TEER) measurements.

Materials and Methods

Cell Culture

The MDCK cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and grown in RPMI 1640 (Hyclone, USA) supplemented with 10 % heat-inactivated fetal bovine serum (Excell, China), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, USA).

The mpkCCD cell line was the kind gift of Prof. Zhiren Zhang (Medical University of Harbin, China) and was grown in 1:1 mixture of Dulbecco modified Eagle medium/F-12 media (Invitrogen, USA) supplemented with 2 g/L glucose monohydrate (Guangfu, China), 1 % (v/v) insulin–transferrin–selenium-G supplement (Gibco, USA), 0.5 mM dexamethasone (Sigma, USA), 1 μM triiodothyronine (Sigma, USA), 10 μg/mL EGF (Peprotech, USA), 15 mM HEPES (Amresco, USA), 2 % heat-inactivated fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin.

Two cell lines were grown at 37 °C in a humidified atmosphere containing 5 % CO_2. They were passaged twice per week with medium changes every 2 days. For experiments, cells were seeded on cell culture inserts (0.4-μm pore size on polyethylene terephthalate track-etched membrane; Becton Dickinson Labware Europe, France) at a density of 1×10^5 cells/well and grown for about 14 days to form a polarized monolayer.

Hopping Probe Ion Conductance Microscopy

HPICM setup was upgraded from a commercial ICnano scanning ion conductance microscope (Ionscope, United Kingdom) as described previously (Liu et al. 2011; Yang et al. 2011, 2012; Novak et al. 2009). Briefly, HPICM was composed of an ICnano scanner controller (Ionscope, UK) and a sample scan head SH01 (Ionscope, UK). The ICnano controller controlled the Z direction LISA piezo (25 μm, P-753.21C; Physik Instrumente, Germany) to perform positioning vertically and hopping of the nanopipette probe. Two PIHera piezo (100 μm, P-621.2C; Physik Instrumente, Germany) controlled the cell movement under the probe in the horizontal X–Y direction during the scanning. An external Axon MultiClamp 700B amplifier (Molecular Devices, USA) was utilized to monitor the ion current flowing into the nanopipette tip and provide a DC voltage of 200 mV between the nanopipette electrode and the reference electrode. When the hopping probe was approaching to the cell surface, a 0.4 % reduction of the reference current was set to maintain the sample–pipette separation. The time required to scan a big area of 40×40 μm was about 20 min, while it took 15 min to perform a 10×10 μm high-resolution scan. All scans were carried out at room temperature (25 ± 2 °C).

Nanopipettes for HPICM were pulled from borosilicate glass (O.D. 1.00 mm, I.D. 0.59 mm, VitalSense Scientific Instruments, China) with a laser-based puller Model P-2000 (Sutter Instruments, USA). In normal HPICM scans, the resistance of nanopipette was about 100 MΩ when the nanopipette was filled with L15 medium. When patch clamp recordings were performed, both nanopipette and bath solution were all changed to a homemade patch solution (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.2).

Patch Clamp Recording

Ion channel currents were recorded with the cell-attachment configuration of the patch clamp technique with a Axon multiclamp 700B amplifier connected to a Digidata 1440A analog-to-digital converter (Molecular Devices, USA). The patch nanopipette was precisely controlled by HPICM to perform while approaching the cell surface. When the nanopipette get close to the cell surface, suction was applied, forming a gigaohm seal and making a successive cell-attached recording. Evoked currents were sampled at 10 kHz and low pass filtered at 1 kHz by an eight-pole Bessel filter. The current data were digitized and stored by the Digidata 1440A interface, then analyzed by ClamPex10.2 and Clampfit 10.2 software (Molecular Devices, USA).

TEER Measurements

TEER measurements of the cell monolayer were performed as previously described (Zhang et al. 2005). Briefly, the TEER of the cell monolayer was investigated with a commercial Epithelial Volt ohmmeter (EVOM2; World Precision Instruments, USA) and an ENDOHM-24 SNAP EndOhm Chamber (World Precision Instruments, USA). The TEER measurements were performed in CO_2-independent L15 medium at physiological temperature (37 ± 1 °C).

Values were presented as mean ± standard error of the mean. Statistical significance was determined by Student's t test. A difference between means at a level of $P < 0.05$ was considered statistically significant.

Results

Topological Images of MDCK and mpkCCD by HPICM

HPICM topographical images of two living mammalian CCD cell monolayers revealed microvilli projections and cell borders (Fig. 1a, c), which were similar to those previously observed from a A6 cell monolayer (Zhang et al. 2005; Conceicao et al. 2003). The average cell diameter of MDCK was about 25 µm—much larger than that of mpkCCD cells, which is about 15 µm.

For observing the fine membrane structures of cell monolayer, zoom-in white-dotted-square-marked 10 × 10–µm regions of two cell monolayers were further scanned at an even higher resolution (Fig. 1b, d). It was observed that the microvilli of MDCK cells were more abundant than those of mpkCCD cells.

TEER Measurements of MDCK and mpkCCD Cell Monolayers

The TEER values of the MDCK and mpkCCD monolayers were 10.5 ± 0.57 and 5.4 ± 0.25 kΩ cm^2, respectively (Fig. 2a). To evaluate the ENaC activity of MDCK and mpkCCD cell monolayers, the specific ENaC inhibitor amiloride was added to block all the sodium ion channels in two CCD cell monolayers. The amiloride-sensitive parts of TEER values were measured by EVOM^2 (World Precision Instruments, USA) before and after amiloride treatment. The results showed that there were no significant differences in amiloride-sensitive TEER of MDCK and mpkCCD cell monolayers (Fig. 2b).

Single Channel Recording of MDCK and mpkCCD

Cell-attached recordings were performed on the cell surface of MDCK and mpkCCD monolayers by HPICM combined patch clamping. Representative single-channel currents of both cells were recorded when the hold potential was 0, −40, and −60 mV (Fig. 3a, b). The amplitude of ion channel currents of two cells were in the range of 0.2–0.5 pA. The single channel conductance of the inward currents was 4.77 ± 0.32 pS ($n = 6$) for MDCK and 3.86 ± 0.23 pS ($n = 6$) for mpkCCD cells. The channel open probability (NP_o) was about 0.23 for MDCK cells and 0.28 for mpkCCD cells at the holding potential of −40 mV. The second-level openings were also recorded from MDCK and mpkCCD cells. According to the current amplitude, opening dwell time, and the channel open probability of ENaC, our recorded single-channel activities were similar to previously reported ENaC currents obtained from both cell lines (Staruschenko et al. 2007; Ishikawa et al. 1998; Karpushev et al. 2010)

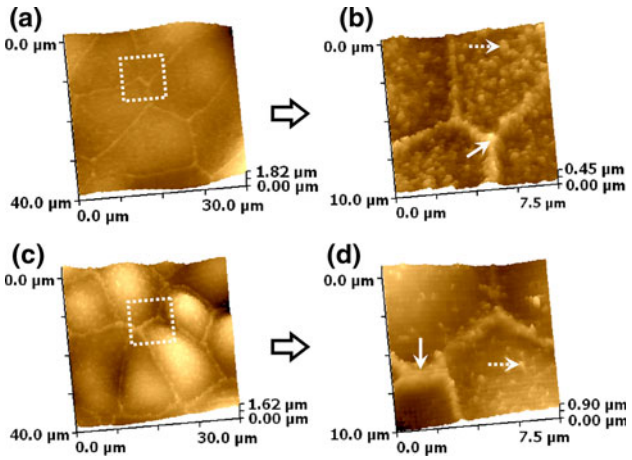

Fig. 1 HPICM topological images of MDCK and mpkCCD. **a** Area of MDCK monolayer. **b** Close-up of the *white-square-marked* area of the MDCK cell monolayer in **a**. **c** Area of mpkCCD cell monolayer. **d** Close-up of the *white-square-marked* area of mpkCCD in **c**

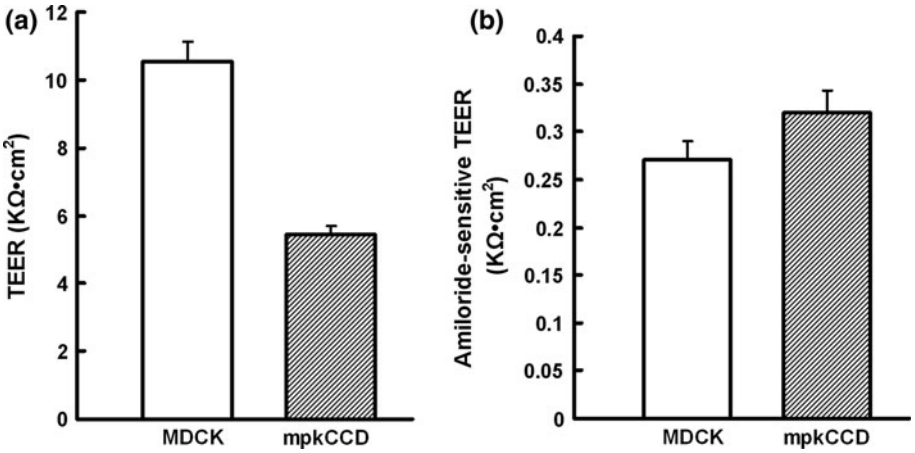

Fig. 2 TEER measurement of MDCK and mpkCCD cell monolayers. **a** TEER of MDCK and mpkCCD monolayers ($n = 6$). **b** Amiloride-sensitive TEER responded to 10 µM amiloride in MDCK and mpkCCD monolayers ($n = 6$)

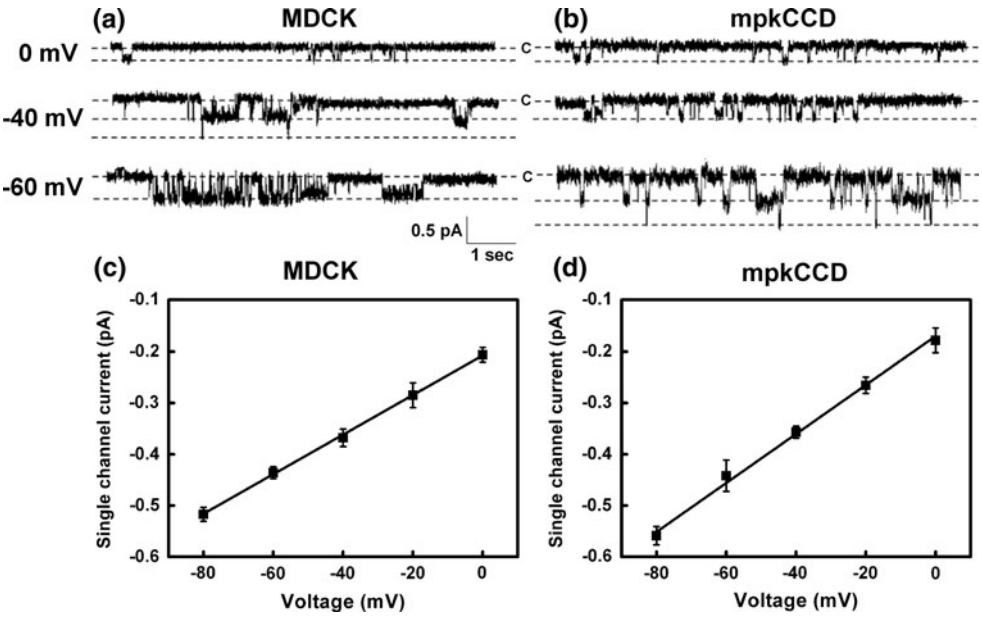

Fig. 3 Cell-attached single channel recordings and current–voltage (*I–V*) curves from MDCK (**a**) and mpkCCD (**b**). Current traces were recorded at voltages held at 0, −40, and −60 mV, respectively. The corresponding *I–V* curves of the cell-attached recordings were obtained at holding potential −40 mV from MDCK (**c**) and mpkCCD (**d**) ($n = 6$)

Discussion

Renal CCD epithelia have a very important physiological function in maintaining the balance of intake and excretion of water and salt; imbalances result in a change in blood pressure (Kamynina et al. 2001; Bubien 2010). Numerous studies have demonstrated that healthy CCD epithelial monolayers are characterized with high TEER, intact tight junctions, and microvilli microstructures (Lesniewska et al. 1998; Rosenthal et al. 2010). Moreover, they are also characterized by functional Na^+ channels, such as amiloride-sensitive and non-voltage-dependent Na^+ channels (Ishikawa et al. 1998). Here, we studied the morphology and electrophysiological function of MDCK and mpkCCD cells. Both MDCK and mpkCCD epithelial cells developed integrating tight monolayers. The high-resolution HPICM images clearly demonstrated fine membrane structures, including microvilli and tight junctions in the cell membrane of MDCK and mpkCCD; these observed membrane structures were similar to those previously imaged with AFM and SEM (Hoh and Schoenenberger 1994; Nascimento et al. 2007). Furthermore, our results indicated that the MDCK and mpkCCD cell lines displayed ENaC activity at both the monolayer level and the single ion channel level.

In summary, the characterization of two typical mammalian CCD cell lines with noncontact HPICM demonstrated that MDCK and mpkCCD cell lines were good mammalian CCD models and may be used for further mechanism research on Na^+ absorption and reabsorption.

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