J. Membrane Biol. 97, 43–51 (1987)

The Journal of
Membrane Biology
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Voltage and Ca^{2+}-Activated K^+ Channel in Cultured Epithelial Cells (MDCK)

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Summary. Patch-clamp techniques were used to study a K channel in the cell membrane of MDCK cells. This cell line derives from the kidney of a normal dog, presumably from the distal nephron, a region involved in potassium secretion. The cells were cultured in confluent monolayers and approached from the apical side. The K channel we describe is Ca^{2+} and voltage activated, has a conductance of 221 ± 7 pS, and can be inhibited by 10 mM tetraethylammonium and by 1 mM quinidine, but not by 4-aminopyridine, nor by 1 mM Ba^{2+} added to the outer side. Using the whole-cell configuration, we find that most of the cationic conductance of the membrane is constituted by a K-specific one (maximum K conductance 32.1 ± 3.9 nS *vs.* a leak conductance of 1.01 ± 0.17 nS). Comparisons of the maximum K conductance with that of a single K channel indicates that an MDCK cell has an average of 145 such channels. The membrane capacity is 24.5 ± 1.4 pF.

Key Words patch clamp · whole-cell clamp · MDCK cells · cultured epithelium

Introduction

MDCK cells, derived from the kidney of a normal dog (Madin & Darby, 1958), can be cultured as monolayers that behave in many respects as natural transporting epithelia (Cereijido et al., 1978). Structural, functional and immunological information indicates that this type of cell resembles those of the distal tubule and early portion of the collecting duct (Lewis & Spector, 1981; Valentich, 1981; Herzlinger, Easton & Ojakian, 1982; Garcia-Perez & Smith, 1983; Hassid, 1983). This part of the nephron is engaged in K^+ secretion and has a variety of K channels (Hunter et al., 1984; Koeppen, Beyenbach & Helman, 1984). Accordingly, we investigated whether MDCK cells have channels specific for this ion. We resort to patch (outside and inside-out) and whole-cell clamp techniques. The use of this approach to study channels in renal cells was recently reviewed by Palmer (1986).

We have worked with monolayers of cells that had been plated at confluence for 4–7 days, a situation where they have tight junctions and have their membrane polarized into an apical and a basolateral domain (Cereijido et al., 1978). Since we approached the cells from their apical side, and this side has microvilli and is coated with glycocalyx, the obtaining of seals that had 10 or more gigaohms was somewhat surprising. We found that some 70% of the patches had a high-conductance K channel, which was voltage and Ca^{2+} dependent. These, as well as other characteristics of this channel, are reported below.

Materials and Methods

Cell Culture

Starter MDCK cultures were obtained from the American Type Culture Collection (MDCK, CCL-34) (Madin & Darby, 1958). Upon arrival cells were cloned and all experiments reported in the present article were performed in cells of Clone 7, chosen because of its intense blistering activity when plated on non-permeable supports. Cells were grown at 36.5°C in disposable plastic bottles (Costar 3250, Cambridge, Mass.) with an air-5% CO_2 atmosphere (VIP CO_2 incubator 417, Lab Line Instruments, New Brunswick, N.Y.) and 20 ml of Dulbecco's modified Eagle's medium DMEM (Grand Island Biological Co., [GIBCO] 430-1600, Grand Island, N.Y.) with 100 U/ml of penicillin, 100 μg/ml of streptomycin (GIBCO 600-5145), 0.8 U/ml of insulin (Eli Lilly, Mexico, D.F.), and 10% fetal calf serum (GIBCO 200-6170); in the following text this complete medium is referred to as CDMEM. Cells were harvested with trypsin-EDTA (In Vitro, Mexico) and plated on glass coverslips contained in 30-mm Petri culture dishes (Linbro Chemical, New Haven, Conn.). Cells were usually between the 60-80th passage and, unless otherwise stated, were studied 4–7 days after plating at confluence.

Electrical Recordings

Monolayers of cells cultured on coverslips were deposited on the glass bottom of a flat chamber fixed to the stage of an inverted

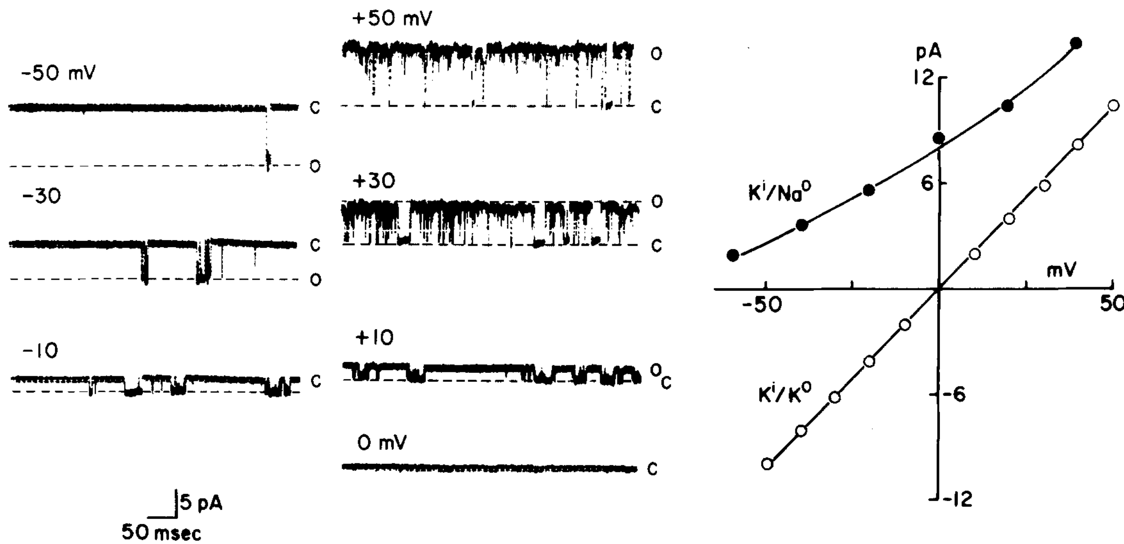

Fig. 1. *Left:* Ionic currents recorded with a single patch clamp in the outside-out configuration from the apical side of a MDCK cell 6 days after plating. Both sides of the membrane were in contact with a solution containing 75.5 mM K_2SO_4 as main salt and 2×10^{-6} M Ca^{2+} (buffered with EGTA). Holding membrane potentials are indicated at the left-hand side of each recording and correspond to the inside of the glass pipette. *c* and *o*: Levels of current at which the channel is closed and open, respectively. *Right: I/V* curves of single channels. Open circles correspond to the same channels of the recordings on the left side of the figure. Filled circles correspond to a different outside-out patch clamp in which the K^+ of the bathing solution was replaced by Na^+

Diavert microscope (Leitz, Wetzlar) equipped with Hoffman optics. Ionic currents were studied in the several configurations described by Hamill et al. (1981): patch-clamp (inside-out and outside-out) as well as whole-cell clamp. Recordings were made through a Dagan 8900 amplifier (Minneapolis, Minn.), using micropipettes pulled from borosilicate glass (Corning, 1.1–1.2 i.d.) using a vertical pipette puller (David Kopf Instruments 700C; Tujunga, Calif.). Micropipettes were covered with Sylgard and fire polished and had a resistance of 3–4 MΩ. They were mounted in a pipette holder attached to a Narishige, MF83 (Tokyo, Japan) hydraulic micromanipulator. All recordings reported were obtained after gigaseals of at least 10 GΩ. Sealing and recordings were monitored with a Tektronix 5115 oscilloscope (Beaverton, Ore.). Currents were recorded through Ag-AgCl electrodes and filtered at 1 KHz. Single-channel currents were recorded with a digital audio processor (Sony, Japan) and stored in a videocassette with a video recorder (Sony, Japan). Signals were digitalized at 1 KHz and acquired and analyzed with a Compaq PC computer using a Fetchex program (Axon Instrument, Burlingame, Calif.). Stimulation and acquisition in whole-cell clamp studies were made with a Clampex 1 program (Axon Instruments). Analysis was performed using a Clampan 1 program (Axon Instruments). Displays were made with an Epson FX-80 Printer (Torrance, Calif.) or with pictures taken with a Polaroid camera (Cambridge, Mass.) from the oscilloscope screen.

Experiments were performed at room temperature (20–25°C). Unless otherwise stated, the intracellular solution contained (in mM): 154, methanesulfonic acid; 10, NaOH; 141, KOH; 1.54, $Ca(OH)_2$; 1.0, $Mg(OH)_2$; 2.3, EGTA; 10, glucose; 10, HEPES-KOH (pH 7.4), with a free-Ca concentration of 3×10^{-7} M. This solution is referred to as *K rich*. The extracellular solution contained (in mM): 154, methanesulfonic acid; 146, NaOH; 5, KOH; 2.0, $Ca(OH)_2$; 1.0, $Mg(OH)_2$; 10, glucose; 10, HEPES-NaOH (pH 7.4). The volume of the bathing solution was 1.5 ml and was changed through perfusion.

Cell-to-Cell Communication

The micropipette used for whole-cell studies was filled with a solution containing a 50 : 50 mixture of two Ringer solutions containing (in mM): (i) 165, LiCl; 10, HEPES/Tris; (pH 7.4) and (ii) 151, KCl; 0.01, $CaCl_2$; 1, $MgCl_2$; 10, HEPES/Tris; 10, glucose; 1.92, EGTA (pH 7.4); free Ca^{2+} concentration $<10^{-9}$ M, and 1% Lucifer Yellow CH (Polyscience, Warrington, Pa.). Once the gigaseal was obtained, the patch was bursted and the dye penetrated the cell in less than 30 sec without detectable leakage to the bathing medium. The inverted microscope was equipped with epifluorescence optics. Pictures were taken with an Orthomat camera (Leitz, Wetzlar).

Results are expressed as mean ± SE (number of observations).

Results

Patch Clamps

Although MDCK cells have microvilli and glycocalyx on their apical regions (Cereijido et al., 1978), these do not prevent the obtaining of seals around 10-30 GΩ without resorting to special treatment of the cell surface with proteolytic enzymes.

Figure 1, on the left-hand side, shows a series of recordings obtained with the outside-out configuration using a K-rich solution on both sides of the membrane. The potential at which each recording was made is written on their upper left side, and the

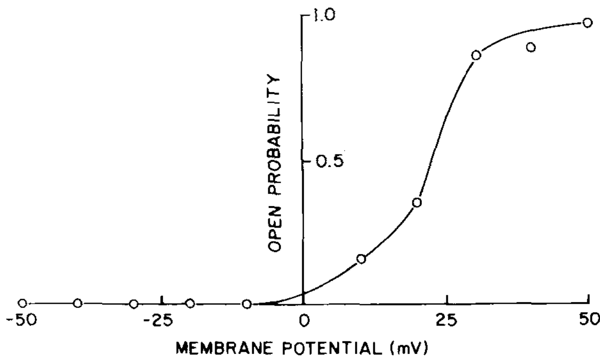

Fig. 2. Relationship between membrane potential and open probability of a single channel, as studied in the inside-out configuration. Membrane potential refers to the cytoplasmic side of the membrane. Both sides contain a K-rich solution (75 mM K_2SO_4). The concentration of Ca^{2+} on the inside was 2×10^{-6} M and on the outside was lower than 10^{-9} M

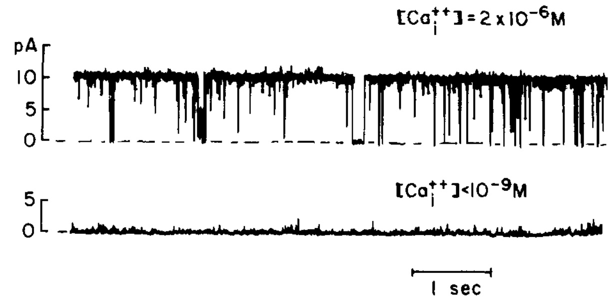

Fig. 3. Effect of calcium concentration on the inside of the membrane of an inside-out patch. Holding potential was 50 mV. (cytoplasmic side +) *Upper recording:* Ca^{2+} concentration, 2×10^{-6} M. *Lower recording:* Made with the same patch after several washes with a solution containing less than 10^{-9} M Ca^{2+}

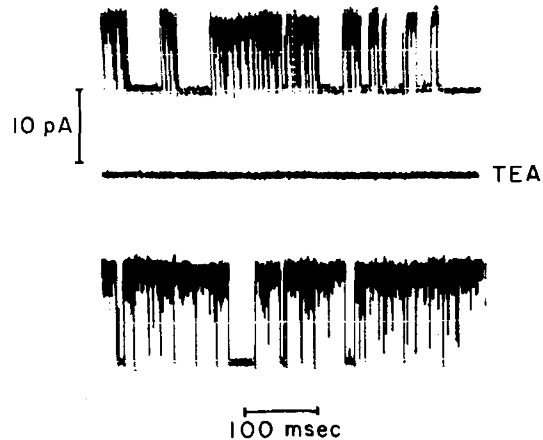

Fig. 4. Effect of tetraethylammonium (TEA) on the ionic current of a channel in the outside-out configuration. Both sides of the membrane were in contact with a K-rich solution. Ca^{2+} concentration was 2×10^{-6} M. Holding potential was 50 mV (cytoplasmic side +). The upper and the bottom recordings were obtained under control conditions before and after a change to a bathing solution containing 10 mM TEA (middle)

closed and open states are indicated by the letters *c* and *o*, respectively. When the voltage is negative on the cytoplasmic side (e.g. −50 mV) opening events are very scarce and current flows towards the cytoplasmic side. As the voltage is set at less negative values, the size of the pulses decreases. Current is negligible at zero millivolts and reverses when the cytoplasmic side is made positive. The current/voltage relationship on the right-hand side of Fig. 1 shows (open circles) that the curve described with K-rich solution on both sides is straight, symmetric and has a slope of 204 pS. In a series of five similar *I/V* curves we found an average conductance of 221 ± 7 pS.

If K^+ is replaced by Na^+ in the bathing solution (filled circles) there is a positive current flowing from the pipette to the bath. Since both solutions have a similar anionic concentration, this indicates that the channel has a strong K-over-Na selectivity.

Figure 1 also shows that, as the voltage on the cytoplasmic side is made first less negative and then positive, opening events become more frequent until the channel spends most of the time in the open state. This is also illustrated in Fig. 2, which was obtained with recordings of 50 to 60 sec at each voltage, and indicates that the K channel is voltage dependent.

Figure 3 shows the results obtained with a patch clamp in the inside-out configuration. The upper trace was obtained in the presence of 2×10^{-6} M Ca^{2+} (buffered with EGTA) and the lower one with less than 10^{-9} M on the cytoplasmic side. In both cases the membrane potential was clamped at 50 mV (cytoplasmic side +). Even when at this potential the probability of finding the channel open is near 1.0 (*see* Fig. 2), the removal of Ca^{2+} shifts it to an almost permanently closed state. At this Ca^{2+} concentration, K channels are only occasionally observed, even at a clamping potential of 70 mV (*data not shown*).

To further characterize this potassium channel we tested the effect of several inhibitors. Figure 4 corresponds to an outside-out patch clamped at 50 mV (cytoplasmic side +) and bathed on both sides with a K-rich solution containing SO_4^{2-} as main anion, where tetraethylammonium (10 mM) provokes a complete but reversible blockade of the channel. Figures 5 and 6 correspond also to outside-out patches with K-methanesulfonate in the pipette and Na-methanesulfonate in the bath as main salts. Each recording is a segment of a 1-min tape record-

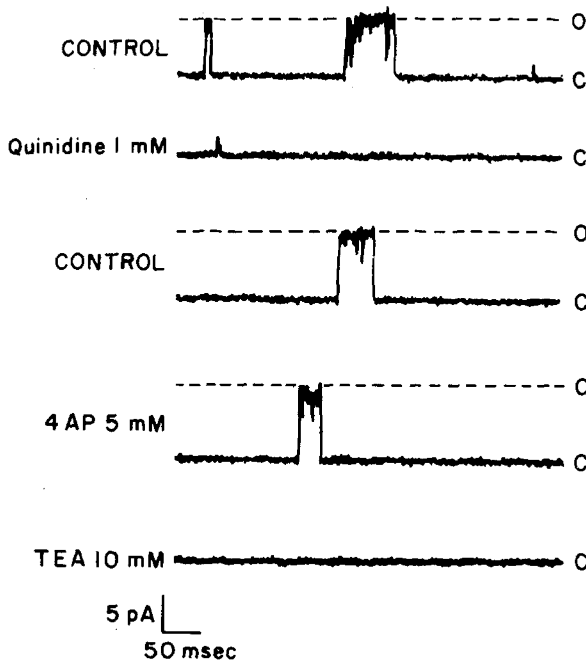

Fig. 5. Effect of several inhibitors on the ionic current of a single channel, as studied in the outside-out configuration. The solution in the pipette was K rich (141 mM K-methanesulfonate) and contained 3×10^{-7} M Ca^{2+}. The solution bathing the outside was rich in Na^+ (146 mM as methanesulfonate salt). Holding potential was 20 mV (cytoplasmic side +). Changes were made in the solution bathing the extracellular side. *o* and *c* correspond to the current levels when the channel is closed or open, respectively

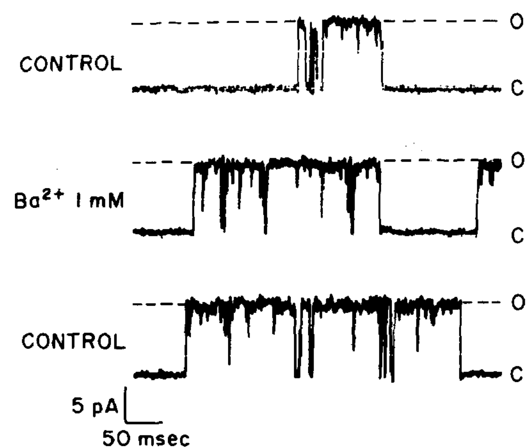

Fig. 6. Fragments taken from 1-min tape recordings, showing the lack of effect of 1.0 mM Ba^{2+} on the extracellular side. Recordings were made with the outside-out configuration in a MDCK cell 4 days after plating. Solutions and holding potential were as in Fig. 5. The upper and the lower controls were obtained before and after the recording with Ba^{2+}

ing. Quinidine (1 mM) produces a reversible inhibition, but the channel is not affected by 5 mM 4-aminopyridine nor by the presence of 1.0 mM Ba^{2+} at the holding potential used.

Whole-Cell Clamp

The whole-cell configuration was obtained by breaking the membrane patch under the seal by suction. Under this condition the cell interior achieves rapid equilibrium with the solution in the pipette (Hamill et al., 1981; Fenwick, Marty & Neher, 1982). We confirmed this rapid penetration by observing the diffusion of Lucifer Yellow from the pipette to the cytoplasm (*see below*). While this technique offers the advantage of avoiding the leakage around the tip of conventional (impaling) microelectrodes, it presents limitations due to potential cell-to-cell couplings (Palmer, 1986). Monolayers of MDCK cells only present this type of communication during a brief period between the 4th and the 20th hour after plating at confluence. This was demonstrated by Cereijido et al. (1984) by the transfer of Lucifer Yellow (using conventional glass microelectrodes) and by the presence of the typical image of gap junctions in freeze-fracture replicas. In the present study we have used cells cultured for 4-7 days, which should not have this type of connection. This was confirmed in a series of studies where the microelectrode was filled with Li-K-rich medium containing 1% Lucifer Yellow CH. The penetration of the dye, continuously monitored with epifluorescence optics in the inverted microscope, was detected in less than 30 sec after bursting the patch (Fig. 7). In only one (out of 19 injected cells) was the dye observed to pass to a neighboring cell. Furthermore, the time course of variation of transmembrane current provoked by a step pulse of voltage can be described by a single exponential term (Fig. 11). This result indicates that cell-to-cell connections may not prevent the maintenance of a spatially homogeneous voltage clamp of MDCK cells cultured in mature (4–7 days old) monolayers.

As shown in Fig. 8, MDCK cells can be whole-cell clamped for, at least, as early as 2 hr and as late as 2 weeks after plating at confluence. Recordings in this and following figures were obtained from a holding potential of −80 mV, and the application of a train of depolarizing voltage steps, alternated with hyperpolarizing steps of one quarter of their values. Only positive current curves were observed. The size of these currents exhibited a considerable variation from one cell to another (Fig. 9). Since the cells used in this study were previously cloned, this

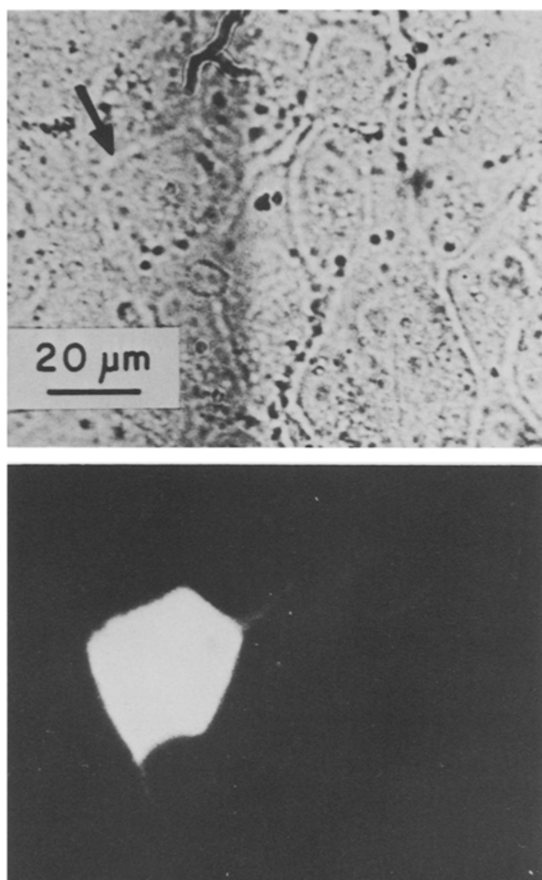

Fig. 7. Intracellular injection of Lucifer Yellow CH in an MDCK cell, using the whole-cell clamp technique. *Above:* Light-field photograph showing a continuous monolayer four days after plating at confluence, with an arrow pointing to the injected cell. *Below:* Epifluorescent image of the same field shown in the upper picture, 1 min after injection of the dye

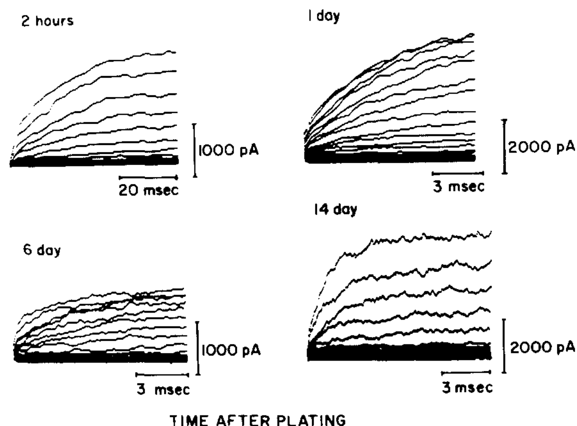

Fig. 8. Recording obtained with the whole-cell clamp configuration in four different MDCK cells that were cultured for 2 hr, 1, 6 and 14 days. Solutions inside the pipette and in the bathing solutions are similar to those in Fig. 5

Table. Electrical parameters of MDCK cells, measured by the whole-cell voltage-clamp configuration

Maximum K^+ conductance (nS)	32.1 ± 3.9
Leak conductances (nS)	1.01 ± 0.17
Capacity (pF)	24.5 ± 1.4
Maximum K^+ conductance ($\mu S/\mu F$)	1360 ± 190
Leak conductance ($\mu S/\mu F$)	39.6 ± 5.3
n	13

Parameters were measured in MDCK cells 4 to 7 days after plating. Intracellular solution was K^+ methanesulfonate rich, with $[Ca^{2+}] = 3 \times 10^{-7}$ M, and extracellular solution (bath) was Na^+ methanesulfonate rich. Cells were voltage clamped at −80 mV and a train of pulses from −120 to +80 mV were passed. Maximum K^+ conductance was obtained from the slope of the linear portion of the voltage-activated currents. Leak conductance was obtained from the fraction of the *I/V* curves, which are not activated by voltage.

variation may not be due to heterogeneity in their population, but must reflect other aspects (e.g. the stage of the cell cycle). In all cases the current was observed to flow from the cytoplasm to the extracellular solution and was activated at depolarizing voltages.

Figure 10 illustrates three recordings obtained in the same cell in which the effect of 10 mM TEA was tested between two control runs. Although this substance was added to the upper (apical) surface, it should quickly reach the basolateral side, because monolayers grown on nonpermeable supports, as in the present case, have most of their tight junctions open. This was demonstrated by labeling Na-K-pumps and other proteins present in the basolateral surface with ^{3}H-ouabain and with ^{125}I plus lactoperoxidase added to the apical side (Cereijido et al., 1981; Meza et al., 1982). Recordings in Fig. 10 clearly indicate that most of the current is blocked by this specific inhibitor of K channels.

The Table summarizes the results obtained with the whole-cell clamp configuration. While the K conductance, measured by the maximal slope of the current/voltage curve, averaged 32.1 ± 3.9 (13) nS per cell, the leak conductance was only 1.01 ± 0.17 nS. The value of the membrane capacity 24.5 ± 1.4 pF is considerably lower than the value of 45.1 ± 2.9 (63) pF obtained with conventional impaling microelectrodes in the noncloned population of cells (Stefani & Cereijido, 1983). This difference may be

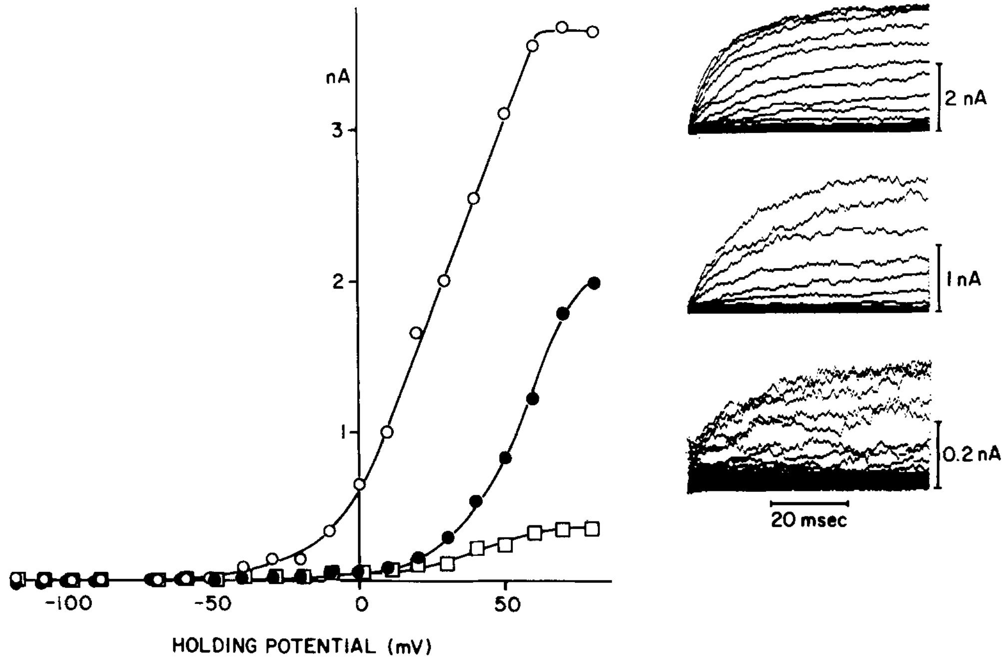

Fig. 9. *Right:* Recordings obtained with the whole-cell clamp configuration in three different MDCK cells. *Left: I/V* curves corresponding to the three sets of recordings shown on the right-hand side. Notice: (i) the wide variability in the ionic current at a given membrane potential; (ii) that in all cases these are out-going currents; (iii) that activation occurs at depolarizing voltages; (iv) that the voltage at which activation occurs is variable; and (v) that the value of the maximal currents achieved in the different cells at a given voltage varies roughly over an order of magnitude

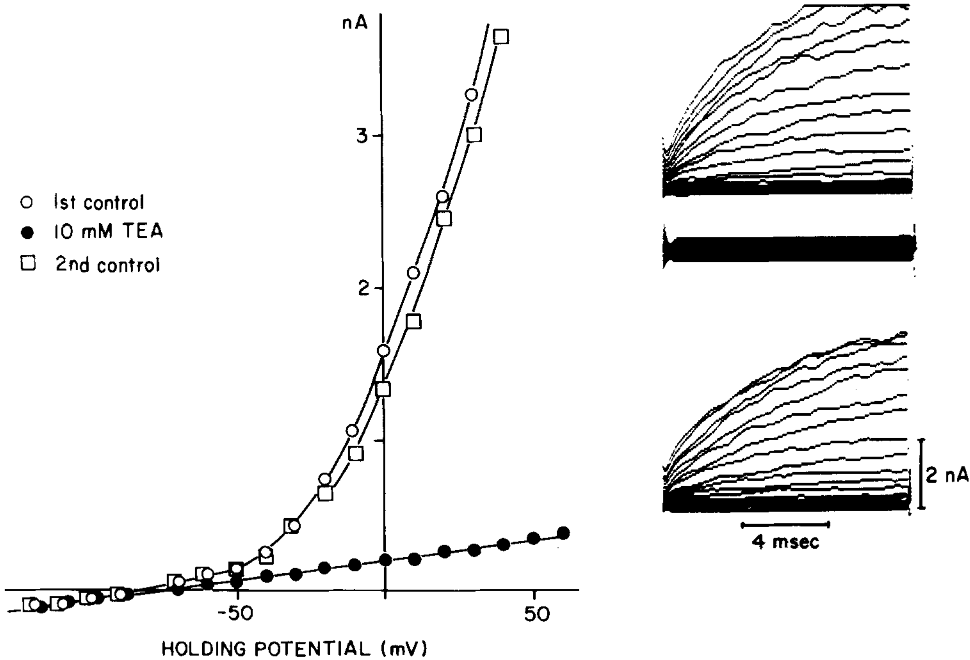

Fig. 10. *Right:* Recordings obtained with the whole-cell clamp configuration of a MDCK cell 1 day after plating. Solutions as in Fig. 5. The upper and bottom recordings are controls obtained before and after changing the bathing solution to one containing 10 mM TEA. *Left: I/V* curves corresponding to the right-hand side

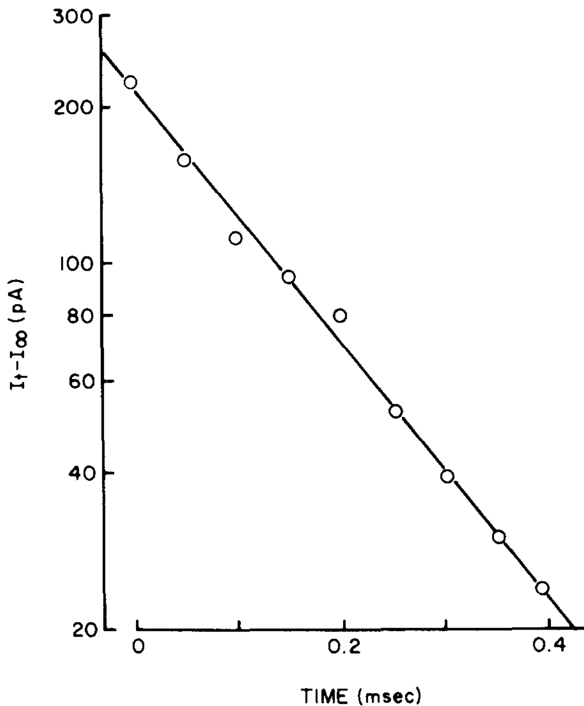

Fig. 11. Time course of the variation of transmembrane current provoked by a step pulse of voltage, from a holding potential of −80 mV, to a new one of −75 mV. Notice that the points can be fitted by a single exponential term. Curves used to compute membrane capacity through the integration of the area under the curve were described by single exponential terms like the one illustrated in this figure

attributed to leak conductance around conventionally impaled microelectrodes. If one square centimeter of cellular membrane can be assumed to have a capacity of 1 μF, the values found can be converted to 1360 $\mu S/cm^2$ for the K conductance and 39.6 $\mu S/cm^2$ for the leak.

Discussion

Because of the many membrane similarities with the distal nephron, MDCK cells are thought to derive from this region of the kidney (Lewis & Spector, 1981; Valentich, 1981; Herzlinger et al., 1982; Garcia-Perez & Smith, 1983; Hassid, 1983). This region is the major site of potassium secretion by the kidney, and the information available indicates that the mechanism involved is passive and located in the apical membrane. Apical K channels with conductances ranging from 4 to 133 pS have been found in renal epithelia of the thick ascending limb of Henle's loop (Guggino et al., 1985) and the cortical collecting tubule (Hunter et al., 1984; Koeppen et al., 1984). Furthermore, Brown and Simmons (1982) observed that MDCK cells have a Ca-activated passive flux of K^+. In keeping with these observations, the results reported in this article demonstrate the presence of a Ca^{2+} and voltage-activated K channel of high conductance in the cell membrane of MDCK cells. This type of channel was first observed in the adrenal chromaffin cells (Marty, 1981) and later in cells of muscle, nerve, endocrine and exocrine glands (Wong, Lecar & Adler, 1982; Atwater, Rosario & Rojas, 1983; Maruyama et al., 1983; Findlay, 1984; Petersen & Maruyama, 1984). MDCK cells also have a potassium conductance sensitive to barium (Paulmichl, Gstraunthaler & Lang, 1985). However, we were unable to detect an inhibitory effect of external barium in the channel reported in the present article. We found instead that the K channel is blocked by TEA and by quinidine, but not by 4-aminopyridine.

The monolayer formed by MDCK cells constitutes a Cl^- secretory epithelium (Brown & Simmons, 1982) and the apical region of these cells contains an anion-selective channel (Kolb, Brown & Murer, 1985). The operation of this anion channel, together with the K-channel described in the present work, may afford the basic mechanisms for the secretion of potassium. Large K channels of this type may also constitute a negative feedback to sustain the membrane potential (Latorre & Miller, 1983; Findlay, 1984; Petersen & Maruyama, 1984). As observed in our whole-cell studies using nonpermeable anions, the membrane conductance remaining upon addition of TEA is a very small fraction of the total conductance. This indicates that passive movements of Na^+ occur mainly through electrically neutral mechanisms (*see* Fernandez-Castelo et al., 1985), and that the K channel may participate in the regulation of membrane voltage.

The value of the maximal K conductance found in whole-cell studies varies considerably from one cell to another. Different groups of MDCK cells have been shown to exhibit different structural aspects (Valentich, 1981) and functional characteristics, such as transcellular resistance (Barker & Simmons, 1981; Richardson, Scalera & Simmons, 1981) and production of specific prostaglandins (Lewis & Spector, 1981). However, the cells used in the present study belong to the same clone. Therefore it is more likely that the large variability in total conductance (*see*, for instance, Fig. 9) would reflect different stages of the membrane during the cell cycle.

Considering that the maximal K conductance we found in whole-cell studies is 32.1 ± 3.9 nS and

that the average K conductance of the channel is 221 ± 7 pS, a total of 145 K channels per cell can be calculated. Assuming that the cell membrane has 1 cm^2 per μF, the 24.5 ± 1.4 pF per cell can be converted into 2450 μm^2 per cell. If the 145 K channels were homogeneously distributed over this surface, there would be a channel per 16.9 μm^2. Since the patch of membrane clamped has an area of around 1 μm^2, one would expect to observe a K channel every 16.9 gigaseals. However, we observe this type of channels in roughly 70% of the successfully gigasealed patches. This suggests that the apical membrane of MDCK cells has a channel density 12 times higher than expected on the basis of random distribution over the entire surface of the cell. The observed distribution will be compatible instead with the population of K channels being restricted to the apical region, this region having one tenth of the total surface of the cell. However, if under physiological conditions (i.e., without the symmetrically high K^+ concentration used in the present work) the channel conductance were lower, and if, furthermore, because of the low intracellular free-Ca^{2+} concentration the channels were not fully activated, their number then will be much higher, indicating that they might not be restricted to the apical domain.

We wish to acknowledge the kind advice and criticism of Prof. E. Stefani. This work was supported by research grants from the National Institutes of Health of the U.S. (DK 26481), CONACyT, Zevada Foundation and COSNET of México, and COSBEL, S.A.

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Received 10 October 1986; revised 22 January 1987