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Characterization of Whole Cell Chloride Conductances in a Mouse Inner Medullary Collecting Duct Cell Line mIMCD-3

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Abstract. The chloride conductance of inner medullary collecting duct cells (mIMCD-3 cell line) has been investigated using the whole cell configuration of the patch clamp technique. Seventy-seven percent of cells were chloride selective when measured with a NaCl-rich bathing solution and a TEACl-rich pipette solution. Seventy-five percent of chloride-selective cells (90/144) had whole cell currents which exhibited an outwardly-rectifying (OR) current-voltage (*I/V*) relationship, while the remaining cells exhibited a linear (L) *I/V* relationship. The properties of the OR and L chloride currents were distinct. OR currents (mean current densities at ±60 mV of 66 ± 5 pA/pF and 44 ± 3 pA/pF), were time- and voltage-independent with an anion selectivity (from calculated permeability ratios) of SCN^- (2.3), NO_3^- (1.8), ClO_4^- (1.7), Br^- (1.7), I^- (1.6), Cl^- (1.0), HCO_3^- (0.5), $gluconate^-$ (0.2). Bath additions of NPPB, flufenamate, glibenclamide (all 100 μM) and DIDS (500 μM) produced varying degrees of block of OR currents with NPPB being the most potent (IC_{50} of approximately 50 μM) while DIDS was the least effective. Linear chloride currents had similar current densities to the OR chloride currents and were also time- and voltage-independent. The anion selectivity sequence was SCN^- (2.5), NO_3^- (1.9), Br^- (1.4), I^- (1.1), Cl^- (1.0), ClO_4^- (0.5), HCO_3^- (0.5), $gluconate^-$ (0.3). In contrast to the OR conductance, glibenclamide was the most potent and DIDS the least potent blocker of L currents. An IC_{50} of >100 μM was observed for NPPB block. Neither OR of L chloride currents were affected by acutely or chronically increased intracellular cAMP and were not affected when intracellular Ca^{2+} levels were increased or decreased. The molecular identity and physiological role of OR and linear currents in mIMCD-3 cells are discussed.

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Key words: Inner medullary collecting duct — mIMCD-3 cell-line — Whole cell configuration — Patch Clamp — Chloride conductance — CFTR — Chloride secretion

Introduction

The inner medullary collecting duct (IMCD) is the final location for determining urinary composition (Na^+, K^+ and acid-base balance). Of special interest is the role that the IMCD plays in the regulation of NaCl balance. This segment is the cellular target of atrial natriuretic hormone which promotes marked natriuresis and diuresis (Rocha & Kudo, 1990*a,b;* Terada et al., 1991). In addition several other hormones and paracrines such as vasopressin and prostaglandins act on this segment (Rocha & Kudo, 1990*a,b;* Zeidel, 1993). There is increasing interest and progress in elucidating the cellular mechanisms of transepithelial ion transport in IMCD cells (Rocha & Kudo, 1990*a,b*). It is now established that electrogenic Na^+ reabsorption occurs across the inner medullary duct epithelia and involves an amiloride-sensitive Na conductance (Rocha & Kudo, 1990*b;* Zeidel 1993). Atrial natriuretic hormone not only inhibits net Na^+ absorption in inner medullary collecting duct epithelia by inhibition of the amiloride-sensitive pathway (Rocha & Kudo, 1990; Zeidel, 1993), but also produces a net secretory flow of Na^+ (Rocha & Kudo 1990*a;* Zeidel, 1993). While a considerable body of information exists concerning the nature of Na^+ transport pathways in inner medullary collecting duct (Zeidel, 1993) much less is known about the nature of transcellular Cl^- movements. Both vasopressin and atrial natriuretic hormone stimulate bath to lumen Cl^- flux in inner medullary collecting duct (Rocha & Kudo, 1990*b*). The baseline passive permeabilities for both Na^+ and Cl^- calculated from

isotopic bath to lumen fluxes, are low (circa 1×10^{-5} cm/sec^{-1}) indicating that this segment is quite impermeable to these ions (Rocha & Kudo, 1990*b*); thus transcellular fluxes of both Na^+ and Cl^- will be of primary importance in determining the magnitude and direction of net transepithelial fluxes (Rocha & Kudo, 1990*b;* Zeidel, 1993; Schwiebert, Lopes & Guggino, 1994). To understand the nature of transepithelial Cl^- movements, including secretions, consideration must be given to the nature of Cl^- conductances and Cl^- channels expressed in renal epithelial cells. Molecular techniques have identified a number of Cl^- channels (or Cl^--channel receptors) which show renal expression and where data on specific nephron segmental patterns of expression may exist. These include CFTR (Morales et al., 1994; Schwiebert et al., 1994), a novel splice variant of CFTR (Morales et al., 1994; Schwiebert et al., 1994), CLC Cl^- channels (Uchida et al., 1992, 1995; Adachi et al., 1994) and pI_{CLn} (Paulmichl et al., 1992). The physiological role of such Cl^- channel expression is not yet certain (Schwiebert et al., 1994), although CFTR-like channel activity have been shown to be associated with the apical membrane (and transepithelial Cl^- secretion) in both primary cultures of rabbit distal bright convoluted tubule (Poncet et al., 1994) and in an inner medullary collecting duct cell line (Husted et al., 1995).

Importantly, the availability of IMCD cells that retain significant differentiation in vitro has allowed reconstitution of functional epithelia in vitro and the application of patch clamp studies to study the nature of ion channels in IMCD cells (Sanson et al., 1994; Husted et al., 1995). Rauchman et al., (1993) have described a clonal, osmotically tolerant, inner medullary collecting duct cell line (mIMCD-3) established from a microdissected, terminal, inner medullary collecting duct, from a mouse transgenic for the early region of SV40. This cell-line retains features typical of this segment, for instance an amiloride-sensitive net Na absorption inhibited by atrial natriuretic hormone (Rauchman et al., 1993).

The purpose of the present study was to investigate the nature of the chloride conductance in IMCD cells using the whole cell configuration of the patch clamp technique. We present evidence that IMCD-3 cells possess two separate Cl^- conductances, on the basis of their biophysical properties and sensitivity to pharmacological agents. A preliminary report of the present data has been presented (Shindo, Simmons & Gray, 1995).

Materials and Methods

Cell Culture

mIMCD-3 cells (Rauchman et al., 1993) were kindly provided by Dr. S. Gullans (Brigham and Womens Hospital, Boston, MA). mIMCD-3 cells were routinely cultured (passages 8–20) in Hams F12/DMEM (50/50% v/v) with 1 g/l d-glucose, 2 mM l-glutamine and 60 μg/ml gentamicin supplemented with 10% v/v foetal bovine serum at 37°C in an air/5% CO_2 atmosphere. Stock Roux bottles (75 cm^2 growth area were passaged by trypsinization (2 ml of 0.5% w/v trypsin, 0.7 mM EDTA in Ca/Mg-free saline) to form a cell suspension and culturing at a 1:10 split ratio. For experimental purposes, cells were seeded at 7.5×10^3 cells/cm^2 (for use within 1 day) or at 3.5×10^3 cells/cm^2 (for use at 2–3 days) on to 13 mm diameter glass coverslips for 45 min at 37°C in complete culture medium, washed and then placed in 5 cm diameter petri dishes, in complete growth medium, for subsequent culture. Under these conditions, epithelial colonies of 20–30 cells and larger confluent sheets of cells were present.

Electrophysiology

A glass coverslip containing cells was transferred to a tissue bath (volume 1.5 ml) mounted on a Nikon Diaphot inverted microscope (Nikon, U.K.), and viewed using phase contrast optics. Current recordings were made at 21–23°C from either single cells, cells in small clusters, or from confluent monolayers, using the whole cell configuration of the patch clamp technique (Hamill et al., 1981; Gray, Plant & Argent, 1993). Pipettes were pulled from borosilicate glass (Clarke Electromedical, U.K.) and had resistances, after fire polishing, of between 2.5 and 5 MΩ. Seal resistances were typically between 10 and 30 GΩ.

Whole cell currents were recorded with an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, FRG) using two basic voltage-clamp protocols. (i) During continuous recording, the membrane potential was held at 0 mV and then alternately clamped to ±60 mV for 1 sec. Between each pulse there was a 1-sec interval at the holding potential. (ii) To obtain current-voltage (*I/V*) relationships, the membrane potential was held at 0 mV and then clamped over the range ±100 mV in steps of 20 mV. Each voltage step lasted 500 msec and there was an 800-msec interval at the holding potential between steps. Data were filtered at 1 kHz and sampled at 2 kHz with a Cambridge Electronic Design 1401 interface (CED, Cambridge, UK), and stored on either a digital tape recorder or the computer hard disk.

When constructing *I/V* plots, we have used the average current measured over a 200 msec period starting 180 msec into the voltage pulse. The currents were not leak-corrected. Series resistance (R_s) was typically 2 to 3 times the pipette resistance, and R_s compensation (40 to 70%) was routinely used. Membrane potentials (V_m) have been corrected for current flow (I) across the uncompensated fraction of R_s using the relationship: $V_m = V_p - IR_s$, where V_p is the pipette potential. Junction potentials were measured and the appropriate corrections applied to V_m as previously described (Gray et al., 1990). Reversal potentials (E_{rev}) and conductance data were obtained from *I/V* plots after fitting a second or third order polynomial using least squares regression analysis. E_{rev} was obtained by interpolation, and whole cell conductances have been calculated between E_{rev} and ±60 mV. Anion permeability ratios were derived from E_{rev} values using the Hodgkin-Katz modification of the Goldman equation (Gray et al., 1990), and assumes that anion replacement does not alter any cation conductance in mIMCD-3 cells.

The input capacitance of cells was measured using the analogue circuitry of the EPC-7 amplifier and compensated prior to the start of recording. Capacitance values were used to calculate current density which is expressed as pA/pF.

Solutions and Chemicals

The standard pipette solution contained (mM): 110.0 TEACl, 2.0 $MgCl_2$, 0.2 EGTA, 1.0 ATP, 10.0 HEPES, pH 7.2 (with NaOH. Total

Na = 9.2 mM, n = 3). The osmolarity of this solution was 230 mOsmoles/kg, and the calculated free calcium concentration was <1 nM. The standard Na-rich bath solution contained (mM): 137.0 NaCl, 0.3 NaH_2PO_4, 5.4 KCl, 0.3 KH_2PO_4, 2.8 $CaCl_2$, 1.2 $MgSO_4$, 5.0 glucose, 14.0 Tris base, pH 7.4 (with HCl). The osmolarity of this solution was 300 mOsmoles/kg. The total Cl^- in the standard pipette and bath solutions were 114 and 161 mM respectively. In the anion-replacement bath solutions, 100 mM of the NaCl in the standard solution was replaced by the 100 mM sodium salt of another anion, or by 185 mM sucrose for the reduced NaCl bath solution. To produce a TEACl bath solution all of the monovalent cations were replaced by TEACl. A low Ca^{2+} bath solution was made by the addition of 1 mM EGTA together with 602.5 μM $CaCl_2$ to give a calculated free Ca^{2+} of 0.1 μM. Bath solution changes were accomplished by gravity feed from reservoirs at a flow rate of 5 to 6 ml/min^{-1}.

The chloride channel blockers 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), flufenamate and glibenclamide (Sigma) were made up as a 10 mM stock solution in dimethylsulfoxide (DMSO) and then diluted to give a final concentration of 1–100 μM. Blockers were freshly made each day in DMSO. The disulfonic stilbene, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and amiloride (both from Sigma) were dissolved directly in the standard bath solution at a concentration of 500 and 50 μM respectively. Cells were stimulated with extracellular ATP (a fresh stock solution of the Na-salt being made in standard bath solution) or a 'cyclic AMP cocktail' consisting of forskolin (1 μM), dibutyryl cyclic AMP (0.1 mM) and isobutylmethylxanthine (0.1 mM) (all from Sigma, Poole, U.K.). All other chemicals were purchased from commercial sources and were of the highest purity available.

Statistics

Significance of difference between means was determined using Student's t test (paired or unpaired). The level of significance was set at $P \leqslant 0.05$. All values are expressed as mean ± SE (number of observations).

Results

Whole Cell Currents from mIMCD-3 Cells

A total of 198 cells were used in this study of which 77% exhibited whole cell conductances that were chloride-selective when measured with a NaCl-rich bathing solution and a TEACl-rich pipette solution (*see* Materials and Methods for details of composition). The remaining 23% of cells had small currents which were not chloride-selective, as assessed by a lack of positive shift in membrane potential upon bathing solution replacement with Na gluconate, and will not be described further. For those chloride-selective cells 75% (90/144) had whole cell currents which exhibited an outwardly rectifying (OR) current-voltage (*I/V*) relationship, while the remaining 54 cells (25%) had whole cell currents which exhibited a linear (L) relationship.

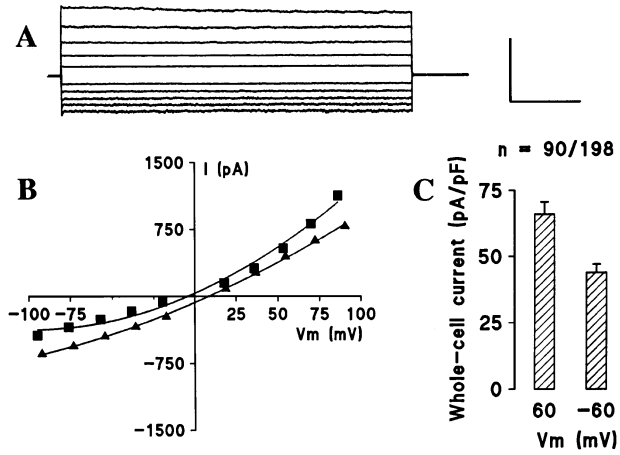

Fig. 1. Characteristics of the outwardly rectifying whole cell currents in IMCD-3 cells. (*A*) Whole cell currents elicited by the ±100 mV protocol described in Materials and Methods. Scale bar 1500 pA and 100 msec. (*B*) Current-voltage (*I/V*) plot for the data in *A*. Squares, *I/V* plot with the standard Na-rich bath solution and TEACl-rich pipette solution. Triangles, *I/V* plot after changing to reduced NaCl solution in the bath (*see* Materials and Methods for details of composition of solutions). (*C*) Summary of whole cell current density measured at E_{rev} ±60 mV. Currents have been normalized to input capacitance which was 15 ± 0.5 pF (n = 90).

Properties of the Outwardly Rectifying Chloride Conductance

Figure 1*A* shows representative whole cell current recordings from a single IMCD-3 cell. The cell was held at a holding potential of 0 mV and then voltage-clamped over the potential range ±100 mV in 20 mV steps (±100 mV protocol). These spontaneously active currents exhibit little voltage- or time-dependence with only a small degree of inactivation at large (>80 mV) potentials. The steady state *I/V* relationship measured under control conditions (Fig. 1*B*, closed squares) shows outward rectification with a reversal potential (E_{rev}) of −4.1 mV, which is close to the equilibrium potential for chloride under these conditions (E_{Cl} −8.6 mV). The mean E_{rev} for the 98 cells was −6.3 ± 0.45 mV. Figure 1*B* (closed triangles) shows the effect of removing 100 mM NaCl from the bath solution and replacing this with an isosmotic equivalent of sucrose, which decreased the outward currents and shifted the E_{rev} to 9.0 mV (E_{Cl} 16.0 mV). The mean E_{rev} from a total of 5 cells was 12.3 ± 1.2 mV (P_{Na}/P_{Cl} = 0.21 ± 0.03). These data confirm that these currents are predominantly chloride-selective. Figure 1*C* shows the mean current density measured at E_{rev} ±60 mV for the 98 cells (with a NaCl-rich bathing solution and a TEACl-rich pipette solution). Overall, the current density was approx 1.5-fold greater at positive potentials (mean values at ±60 mV being 66 ± 5 pA/pF and 44 ± 3 pA/pF respectively). This OR conductance was observed

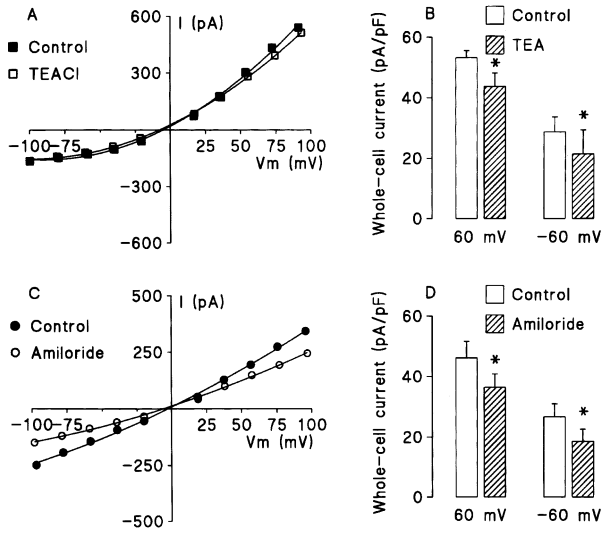

Fig. 2. Effect of bath TEACl and amiloride on characteristics of the outwardly rectifying whole cell currents in IMCD-3 cells. (*A*) and (*C*) Current-voltage (*I/V*) plot for control conditions (with the standard Na-rich bath solution and TEACl-rich pipette solution), and after bath replacement with either TEACl solution or 50 μM amiloride in the standard Na-rich bath solution as indicated. (*B*) and (*D*) Summary of whole cell current density measured at E_{rev} ±60 mV for control conditions and after bath replacement with either TEACl ($n = 4$) or 50 μM amiloride ($n = 8$) as indicated. *Indicates a significant difference ($P < 0.05$).

in single cells as well as cells from confluent monolayers.

Cation Selectivity of Outwardly rectifying Whole Cell Currents

It is apparent from the control E_{rev}s and the shifts in E_{rev} with the reduced NaCl solution (Fig. 1) that the whole cell currents are not ideally Cl^--selective, and probably contain a sodium conductance. We investigated the contribution of a cation conductance by replacing all extracellular monovalent cations with TEA (keeping chloride constant). Figure 2*A* shows that bath TEA-replacement caused a small, negative shift, in E_{rev} from −4.8 to −8.1 mV, and a small reduction in both inward and outward currents which was fully reversible on washout. In paired experiments the mean shift in reversal potential was from −3.3 ± 1.5 mV to −5.6 ± 1.8 mV (n = 4, $P = 0.041$). Current density at E_{rev} ±60 mV for control cells was 53.2 ± 2.3 pA/pF and 28.7 ± 4.9 pA/pF respectively and in the presence of bath TEACl was significantly reduced to 43.7 ± 4.4 pA/pF and 21.4 ± 7.9 pA/pF (Fig. 2*B*). The simplest interpretation of these results is that a significant cation conductance is present in these cells when the bath solution contains small cations (Na & K). Previous experiments have identified amiloride-sensitive Na^+ flux in mIMCD-3 cells (Rauchman et al., 1994) and

Table 1. Anion selectivity of the whole-cell currents of m-IMCD3 cells as determined from shifts in reversal potential

	Control E_{rev}	Experimental E_{rev}	P_x/P_{Cl}
(A) Outward Rectifier			
Anion			
Thiocyanate (4)	−7.5 ± 1.0	−22.3 ± 1.3	2.3 ± 0.2
Nitrate (5)	−7.6 ± 0.7	−18.0 ± 1.3	1.8 ± 0.2
Bromide (11)	−6.5 ± 0.7	−15.4 ± 1.1	1.7 ± 0.1
Perchlorate (11)	−5.3 ± 0.7	−14.4 ± 1.2	1.7 ± 0.1
Iodide (8)	−6.5 ± 1.0	−14.6 ± 1.9	1.6 ± 0.1
Bicarbonate (5)	−2.6 ± 0.7	6.2 ± 1.7	0.5 ± 0.1
Gluconate (11)	−4.6 ± 0.7	12.4 ± 0.9	0.2 ± 0.0
(B) Linear Current			
Anion			
Thiocyanate (4)	−3.5 ± 0.5	−20.5 ± 1.5	2.6 ± 0.1
Nitrate (3)	−2.9 ± 0.8	−14.6 ± 1.6	1.9 ± 0.1
Bromide (4)	−3.8 ± 0.6	−9.3 ± 0.4	1.4 ± 0.8
Iodide (6)	−3.9 ± 0.3	−5.8 ± 0.9	1.1 ± 0.1
Perchlorate (5)	−4.9 ± 1.2	4.6 ± 0.7	0.5 ± 0.1
Bicarbonate (6)	−4.5 ± 1.4	5.7 ± 1.2	0.5 ± 0.1
Gluconate (16)	−4.2 ± 0.6	11.7 ± 0.9	0.3 ± 0.0

Data are expressed as mean ± SEM. Numbers in parentheses are the number of separate observations. P_x/P_{Cl} calculated as described in Materials and Methods.

amiloride-sensitive short circuit current (I_{sc}) in reconstituted epithelia of mIMCD-K2 cells (Kizer, Lewis & Stanton, 1995). We therefore tested the effect of amiloride on OR currents. Addition of 50 μM amiloride to the standard Na^+-rich bath solution also produced a negative shift in reversal potential, as shown in Fig. 2*C* (−4.8 to −8.1 mV), which was reversed on washout. Overall, in 8 separate cells the mean shift in E_{rev}s was from −4.1 ± 0.9 mV to −7.0 ± 0.68 mV ($P = 0.01$), indicating that this cation conductance is blocked by amiloride. In addition, currents were also significantly reduced as shown in Fig. 2*D*.

Anion Selectivity of Outwardly rectifying Whole Cell Currents

Table 1 details the anion selectivity of the OR currents as measured by E_{rev} shifts after substitution of 100 mM bath chloride (keeping Na constant) with the appropriate anion. The permeability ratios were calculated by including a sodium conductance (*see* Materials and Methods). Using the permeability ratios listed in Table 1, the anion selectivity sequence is SCN^- (2.3), NO_3^- (1.8), ClO_4^- (1.7), Br^- (1.7), I^- (1.6), Cl^- (1.0), HCO_3^- (0.5), $gluconate^-$ (0.2).

Effect of Chloride Channel Blockers on Outwardly rectifying Whole Cell Currents

To characterize the OR currents in more detail, we tested the effect of four different compounds that have all been

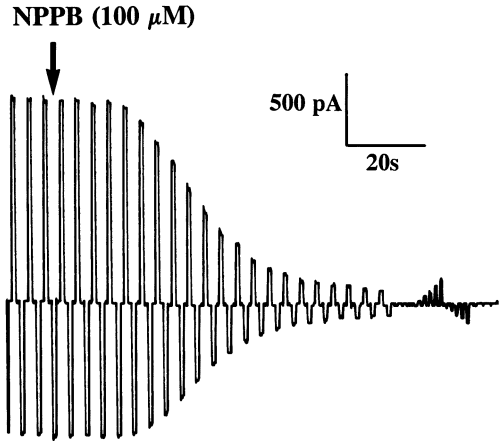

Fig. 3. Continuous recording showing the effect of 100 μM NPPB on outwardly rectifying whole cell currents in IMCD-3 cells. Currents were monitored using the ±60 mV protocol described in Materials and Methods, and blocker was added to the standard Na-rich bath solution as indicated by the arrow.

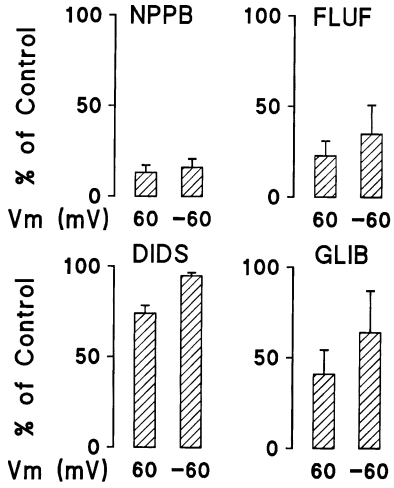

Fig. 4. Effect of chloride channel blockers on outwardly rectifying whole cell currents in IMCD-3 cells. Summary of whole cell current density, measured at E_{rev} ± 60 mV, expressed as a percentage of control value for NPPB, flufenamate (FLUF), DIDS and glibenclamide (GLIB). Data are mean values from 3 (Flufenamate & glibenclamide) or 4 (NPPB & DIDS) separate experiments.

shown to block a variety of Cl^- channels (White & Aylwin, 1990; Chao & Mochizuki 1992; Sheppard & Welsh, 1992; Guggino, 1993). NPPB, flufenamate, glibenclamide (all at 100 μM) and DIDS (500 μM) were tested by adding the blockers to the bath solution. Figure 3 shows the effect of 100 μM NPPB on outwardly rectifying whole cell currents. In this experiment, currents were continuously monitored using the ±60 mV protocol (*see* Materials and Methods). NPPB caused a rapid and large inhibition (80–85%) of whole cell conductance which was voltage-independent. The effect of NPPB was only partially reversed after extensive washout. Figure 4 summarizes the effect of NPPB on current density at E_{rev} ±60 mV. The degree of inhibition was determined from *I/V* plots. Figure 4 also details the amount of block produced by the other three compounds which was determined using a similar protocol. It can be seen that all compounds produced some block with NPPB being the most potent while DIDS the least effective. In fact, DIDS produced no significant block at negative potentials, but did reduce outward currents by 25 ± 10% ($n = 4$).

Regulation of Outwardly rectifying Whole Cell Currents

A striking feature of this study was the spontaneous presence of large Cl^- selective currents in the majority of cells. Since we chose our experimental conditions to prevent any development of swelling-activated Cl^- currents (Solc & Wine, 1991; Frizzell & Morris, 1994), and to inhibit calcium-activated conductances (Gray et al., 1994), it is unlikely that these factors are involved in regulating the outwardly rectifying whole cell currents. We tested the role of calcium further by either activating intracellular Ca^{2+} mobilization with external ATP (0.1 mM) with bath Ca^{2+} at 2 mM (Ecelbarger et al., 1994; Brown, Lang & Simmons, 1995) or by acutely lowering external calcium from 2 mM to 10^{-7} M in the absence of external ATP. Table 2 shows that neither maneuver had any effect on current density. Chloride channels activated by cAMP-dependent protein kinase A have been reported in a number of epithelial cells (Gray et al., 1988, 1993; Fuller & Benos, 1992) including IMCD cells (Husted et al., 1995). We investigated the effect of cAMP on the size of currents by exposing the cells to a mixture of forskolin (1 μM), dibutyryl cyclic AMP (100 μM) and 3-isobutyl-1-methylxanthine (100 μM) for up to 10 min (Gray et al., 1993). As listed in Table 2 this had no significant effect on size of currents. In addition, cells were also preincubated in the cAMP stimulants for up to 120 min prior to whole cell recording, a procedure we have previously shown to stimulate CFTR currents in pancreatic duct cells (Gray et al., 1993), but this again had no effect on current density. We were also unable to increase currents from 8 cells which had no measurable Cl^- conductance with the cAMP 'cocktail.' Taken together, these results indicate that the outwardly rectifying Cl^- conductance is not modulated by either calcium or cAMP.

Properties of the Linear Chloride Conductance

Figure 5*A* shows representative whole cell current recordings for the linear Cl^- conductance using the ±100 mV protocol. These spontaneously active currents ex-

Table 2. Regulation of the whole-cell Cl^- conductances by Ca^{2+} and cAMP

	V_m		Current density		
(A) Outward Rectifier			pA/pF		
Condition			Control		Test
0.1 mM ATP	+60	(3)	50.4 ± 15.1		42.5 ± 15.8
	−60		−33.3 ± 11.0		−38.3 ± 14.6
0.1 μM Ca^{2+}	+60	(4)	80.0 ± 19.1		69.1 ± 30.0
	−60		−58.4 ± 24.1		−59.0 ± 29.3
cAMP 'cocktail'	+60	(5)	77.6 ± 17.3		82.1 ± 16.3
(Acute)	−60		−52.7 ± 16.4		−69.5 ± 14.8
cAMP 'cocktail'	+60	(9)	65.0 ± 10.0	(8)	60.3 ± 13.0
(Prestimulated)	−60		−48.0 ± 5.0		−45.3 ± 10.7
(B) Linear					
Condition			Control		Test
0.1 mM ATP	+60	(3)	110.4 ± 15.4		111.0 ± 9.4
	−60		−109.2 ± 17.3		−122.8 ± 9.8
0.1 μM Ca^{2+}	+60	(3)	89.0 ± 10.4		76.5 ± 18.8
	−60		−87.8 ± 9.8		−69.1 ± 16.6
cAMP 'cocktail'	+60	(4)	64.8 ± 12.4		63.7 ± 11.8
(Acute)	−60		−64.5 ± 13.0		−68.1 ± 11.9
cAMP 'cocktail'	+60	(54)	63.7 ± 4.8	(8)	75.6 ± 14.8
(Prestimulated)	−60		−64.1 ± 5.0		−72.3 ± 14.6

Data are paired from the number of individual experiments given in parentheses, except for data where long-term prestimulation with the cAMP 'cocktail' was performed. In this case data are pooled and are unpaired. Data are mean values ± SEM. ATP was added to the bath and cells exposed for up to 5 min. 0.1 μM Ca^{2+} refers to the calcium concentration of the bath solution. The cAMP 'cocktail' was composed of 1 μM forskolin, 0.1 mM dibutyryl cyclic AMP, and 0.1 mM isobutylmethylxanthine. Prestimulation involved incubations from 60–120 min prior to recording. There were no statistically significant effects between test and control data.

hibited little voltage- or time-dependence over the entire potential range. The steady-state *I/V* relationship measured under control conditions (Fig. 5*B*, closed squares) is linear with a E_{rev} of −4.1 mV. The mean E_{rev} for the 54 cells was −5.1 ± 0.39 mV. Removing 100 mM NaCl from the bath solution and replacing with an isosmotic equivalent of sucrose, (Fig. 5*B* closed triangles) shifted the E_{rev} to 10.3 mV. The mean E_{rev} from a total of 5 cells was 9.9 ± 1.0 mV. ($P_{Na}/P_{Cl} = 0.17 \pm 0.034$). These data confirm that these currents are predominantly chloride-selective. Figure 5*C* shows the mean current density measured at E_{rev} ±60 mV for the 54 cells. These values (64 ± 4.8 pA/pF and 64 ± 5.0 pA/pF) are not significantly different to the OR conductance at +60 mV ($P = 0.70$). This linear Cl conductance was observed in single cells as well as cells from confluent monolayers.

Cation Selectivity of Linear Whole Cell Currents

We tested the effect of bath TEA-replacement on the E_{rev} of linear chloride currents. In 4 paired experiments

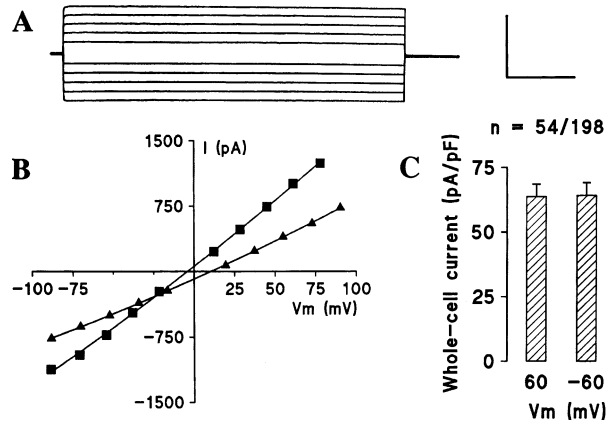

Fig. 5. Characteristics of the linear whole cell currents in IMCD-3 cells. (*A*) Whole cell currents elicited by the ±100 mV protocol. Scale bar 1500 pA and 100 msec. (*B*) Current-voltage (*I/V*) plot for the data in *A*. Squares, *I/V* plot with the standard Na− rich bath solution and TEACl-rich pipette solution. Triangles, *I/V* plot after changing to the reduced NaCl solution in the bath. (*C*) Summary of whole cell current density measured at E_{rev} ±60 mV. Currents have been normalized to input capacitance which was 13 ± 0.5 pF ($n = 54$).

the mean shift in reversal potential was from −5.3 ± 0.4 mV to −9.8 ± 0.9 mV ($P = 0.02$). Current density at E_{rev} ±60 mV for control cells was 41.2 ± 6.6 pA/pF and 41.1 ± 6.7 pA/pF respectively and in the presence of bath TEACl was 35.6 ± 6.4 pA/pF and 36.1 ± 6.7 pA/pF ($P < 0.05$). These results suggest that a significant cation conductance is also present in cells exhibiting linear Cl^- currents as found for cells exhibiting OR currents (Fig. 2*A*). However, in contrast to cells with an OR Cl^- conductance, addition of 50 μM amiloride to the standard Na-rich bath solution produced no significant effect on linear current reversal potential or current density. In 4 separate cells the mean shift in E_{rev}s was from −4.5 ± 0.5 mV to −6.4 ± 0.8 mV ($P = 0.104$); current density at E_{rev} ± 60 mV for control cells was 30.7 ± 5.6 pA/pF and 30.8 ± 5.7 pA/pF respectively and in the presence of bath amiloride was 27.4 ± 5.0 pA/pF and 26.9 ± 4.6 pA/pF ($P > 0.1$). These results therefore suggest that cells exhibiting the linear Cl^- conductance also contain a cation conductance, but this cation conductance displays a different amiloride sensitivity to the one found in OR-containing cells.

Anion Selectivity of Linear Whole Cell Currents

Table 1 details the anion selectivity of the linear currents as measured by E_{rev} shifts after substitution of 100 mM bath chloride with the appropriate anion. Using the permeability ratios listed in Table 1 the anion selectivity sequence is SCN^- (2.5), NO_3^- (1.9), Br^- (1.4), I^- (1.1), Cl^- (1.0), ClO_4^- (0.5), HCO_3^- (0.5), gluconate$^-$ (0.3). This sequence is very similar to the OR conductance apart

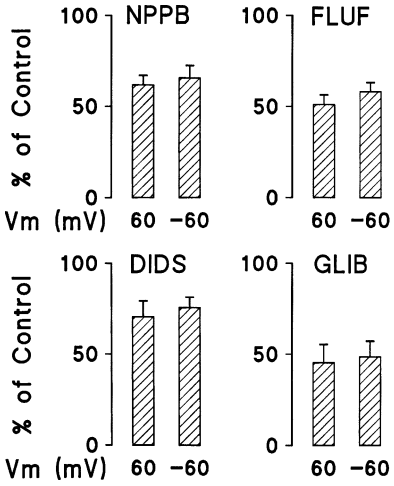

Fig. 6. Effect of chloride channel blockers on linear whole cell currents in IMCD-3 cells. Summary of whole cell current density, measured at E_{rev} ±60 mV, expressed as a percentage of control value for NPPB, flufenamate (FLUF), DIDS and glibenclamide (GLIB). Data are mean values from 3 (Flufenamate & DIDS) or 4 (NPPB & glibenclamide) separate experiments.

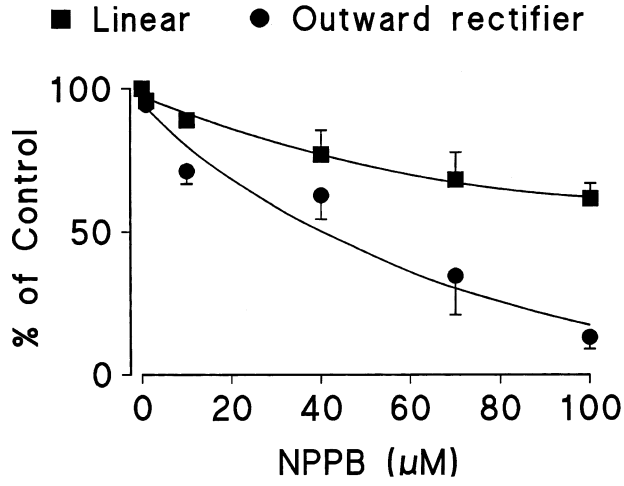

Fig. 7. Dose-response curve for NPPB inhibition of outwardly rectifying (circles) and linear (squares) whole cell currents. Whole cell current density was measured at E_{rev} 60 mV and expressed as a percentage of control value. Each data point represents the mean of 3–4 separate determinations. Lines were fitted by eye.

from the perchlorate permeability, which is markedly lower through the linear conductance.

Effect of Chloride Channel Blockers on Linear Whole Cell Currents

Figure 6 summarizes the effect of NPPB, flufenamate, glibenclamide (100 μM) and DIDS (500 μM) on the linear conductance. The degree of inhibition was determined from *I/V* plots before and after application of the blockers. It is apparent that all compounds produced some block, with glibenclamide being the most potent and DIDS the least potent. When compared to the results obtained for the OR conductance, the overall blocker profile for the linear conductance is quite different. In fact, apart from glibenclamide, a significant difference exists in the amount of block produced by NPPB (at both ±60 mV), flufenamate (at 60 mV) and DIDS (at −60 mV) between the OR and linear Cl^- currents. This difference was particularly marked with NPPB, and the effect was investigated in more detail by employing a range of NPPB concentrations. Figure 7 summarizes the results and shows quite clearly that the linear conductance is much less sensitive to block by NPPB, with an IC_{50} of greater than 100 μM. In contrast, the outwardly-rectifying conductance is blocked by NPPB with an IC_{50} of approximately 50 μM.

Regulation of Linear Whole Cell Currents

Exposing the cells displaying linear Cl^- currents to 100 μM extracellular ATP or to a 10^{-7} M calcium bath solution had no effect on current density (Table 2). In addition, the cAMP stimulants were also without effect on the size of currents. Taken together these results indicate that the linear conductance is also not modulated by either calcium or cAMP.

Discussion

The collecting duct system (cortical collecting duct (CCD), outer medullary collecting (OMCD) and inner medullary collecting duct (IMCD)) is both morphologically and physiologically heterogeneous. Within the rat the inner medullary collecting duct (extending from the junction with the inner stripe of the outer medulla to the papillary tip) the cell population is relatively homogeneous. Clapp et al. (1989) have arbitrarily separated the inner medullary collecting tubule into 3 portions, namely the outer, middle and inner portions (IMCD1, 2 and 3 respectively). IMCD1 may be considered to be a continuation of the OMCD while IMCD2 and IMCD3 display a morphologically homogeneous cell population which have similar physiological characteristics. The physiology of IMCD2 and IMCD3 segments may be considered identical and termed the terminal portion of the IMCD ($IMCD_t$). The cell line used in the present study was established from mice transgenic for the early region of SV40 from a single microdissected portion of the terminal portion of a mouse IMCD (Rauchman et al., 1993).

Physiologically, the terminal portion of the IMCD in the basal state maintains an electrogenic, amiloride-sensitive Na^+ reabsorption dependent on the Na^+-K^+ AT

Pase (Rocha & Kudo, 1990). Atrial natriuretic peptide reduces Na^+ reabsorption as well as stimulating a dose-dependent increase in bath to lumen Cl^- flux in isolated, microdissected, perfused $IMCD_t$ (Rocha & Kudo, 1990*a*). The increase in bath to lumen Cl^- flux was inhibited by bath applied furosemide (Rocha & Kudo, 1990). In addition, dibutyryl cAMP stimulates bath to lumen flux of Cl^- and Na^+ together with a stimulation of a lumen-negative transepithelial voltage. The interdependence of Na^+ and Cl^- fluxes, their dependence upon medium Na^+, K^+ and Cl^- and the blocking action of bath (not lumen)-applied loop diuretic upon Na^+ and Cl^- secretion are all compatible with the ability of the $IMCD_t$ to secrete NaCl in a manner similar to that found in other Cl^- secretory epithelia such as the small intestine and the trachea. (Rocha & Kudo, 1990*a,b;* Frizzell & Morris, 1994). In this model, a basolateral Na^+-K^+-$2Cl^-$ cotransporter accumulates Cl^- above electrochemical equilibrium and Cl^- exists through apically located Cl^- channels, that may be agonist regulated. The existence of Cl^- channels in the basolateral membrane would serve to short-circuit secretory Cl^- movement (*see* Simmons, 1991). An understanding of the nature of Cl^- channels in $IMCD_t$ cells is thus crucial to building models of transepithelial transport in this segment.

The mIMCD-3 cell line used in this study maintains features typical of its tissue of origin including apical to basal Na^+ transport sensitive to inhibition by amiloride and by atrial natriuretic hormone. In addition, mIMCD-3 cells express the secretory form of the $Na^+ + K^+ + 2Cl^-$ cotransporter (from where the mRNA was isolated for cloning and sequencing this transporter (Delpire et al., 1994). Functional studies have shown that the $Na^+ + K^+ + 2Cl^-$ cotransporter is located at the basolateral membrane (Delpire et al., 1994). Patch clamp studies have characterized the K^+ conductances expressed in this cell line (Sanson et al., 1994). mIMCD-3 cells therefore possess transport elements typical of the IMCD, and hence those required to maintain transepithelial salt secretion. In studies of another cell line isolated from terminal IMCD, Kizer et al. (1995) have shown that reconstituted epithelial monolayers maintain electrogenic Na^+ absorption and diphenylamine 2-carboxylate-sensitive electrogenic Cl^- secretion. Similarly, primary cultures of rat papillary cells form epithelia in which electrogenic Na^+ absorption occurs with Cl^- secretion (Husted et al., 1995).

Properties of Whole Cell Cl^- Conductance in mIMCD-3 Cells

In the presence of a Na-rich bath solution and with the pipette solution containing predominantly TEACl the majority (77%) of mIMCD-3 cells show whole cell currents that are Cl^- selective. On the basis of a shift in reversal potential when bath NaCl is replaced by TEA, a component of whole cell current is likely to be due to the presence of a Na^+ conductance (sensitive to amiloride for those cells displaying an outwardly rectifying whole cell conductance). This is consistent with the observation of Na^+ absorption by IMCD epithelia (Rocha & Kudo, 1990*a,b;* Rauchman et al., 1993). The detailed properties of these Na^+ conductances were not examined further.

The magnitude of the whole Cl^- currents in mIMCD-3 cells measured in basal conditions without stimulation is comparable to that observed in pancreatic ductal epithelial cells after stimulation by cAMP (Gray et al., 1993). The characteristics of the whole cell current in the majority of Cl^--selective cells were (i) an outwardly rectifying current/voltage relationship, (ii) time and voltage independence, (iii) block by NPPB, flufenamate, glibencamide but relatively insensitive to DIDS, (iv) an anion selectivity sequence of SCN^- (2.3), NO_3^- (1.8), ClO_4^- (1.7), Br^- (1.7), I^- (1.6), Cl^- (1.0), HCO_3^- (0.5), gluconate$^-$ (0.2). In addition a minor (25%) component of the Cl^--selective cells showed whole cell currents with differing characteristics of (i) a near linear current/voltage relationship, (ii) time and voltage independence, (iii) block by NPPB (low sensitivity compared to OR currents), flufenamate, glibenclamide and DIDS and (iv) an anion selectivity sequence of SCN^- (2.5), NO_3^- (1.9), Br^- (1.4), I^- (1.1), Cl^- (1.0), ClO_4^- (0.5), HCO_3^- (0.5), gluconate$^-$ (0.3).

Whole cell Cl^--currents stimulated by cAMP in rat pancreatic duct cells (due to CFTR) show a linear *I/V* relationship, time and voltage independence, block by NPPB (10 μM) but not by DIDS (500 μM) and an anion selectivity of SCN (4.1), NO_3^- (1.8), Br^- (1.3), Cl^- (1.0), I^- (0.45), HCO_3^- (0.2), ClO_4^- (0.1), gluconate$^-$ (0.07) (Gray et al., 1993). The two different whole cell Cl^- currents recorded in IMCD-3 cells thus show similarities to and differences from those mediated by CFTR currents. It should be noted that the sensitivity of CFTR channels to glibencamide (and noted here for whole cell Cl^- current in mIMCD-3 cells) is not specific for CFTR (Rabe, Disser & Frömter, 1995). The most notable difference between CFTR and mIMCD-3 whole cell Cl^- current is the maintenance of high current densities in the absence of stimulation. Indeed, when cells were acutely or chronically exposed to a 'cocktail' designed to elevate intracellular cAMP, no stimulation of whole cell Cl^- current was observed. Our observations of whole cell Cl^- current in mIMCD-3 cells are different from those of Husted et al. (1995) in rat primary papillary cell epithelium who observe that cAMP stimulates whole cell Cl^- currents. In addition, transepithelial Cl^- secretion (I_{sc}) after amiloride inhibition was inhibited by NPPB and glibenclamide but not by DIDS. Transepithelial Cl^- secretory currents were not inhibited by the loop diuretic bumetanide (Husted et al., 1995). Thus it is clear that

there are differences dependent upon culture type (primary *vs.* cell line) and upon the repertoire of Cl^- conductances and Cl^- transporters accumulating Cl^- above the electrochemical equilibrium expressed in IMCD cells.

Molecular Identity of Whole Cell Cl^- Currents

It seems highly unlikely that the high endogenous level of whole cell Cl^- current in the majority of mIMCD-3 cells represents either Ca^{2+} or volume-activated Cl^- channels since recording conditions were chosen to minimize such currents. The standard pipette solution is Ca^{2+}-free with 0.2 mM EGTA with the calculated free Ca^{2+} being less than 1 nM; in addition, reduction of bath Ca^{2+} to low levels does not affect whole cell Cl^- conductance. The pipette solution was also hypotonic with respect to the bathing solution tonicity, to prevent stimulation of volume-sensitive whole cell current (*see* Materials and Methods). The biophysical characteristics of Ca^{2+}-activated currents, namely time-dependent activation and inactivation at depolarizing and hyperpolarizing potentials (Gray et al., 1994) and swelling-induced Cl^- currents (Solc & Wine, 1991) are also dissimilar to mIMCD-3 whole cell Cl^- current. Ca^{2+} activated Cl^- currents and swelling-induced Cl^- currents are also sensitive to inhibition by low concentrations of stilbenes such as DIDS and DNDs (Anderson & Welsh, 1991; Solc & Wine, 1991; Frizzell & Morris, 1994). Whole cell Cl^- currents in mIMCD-3 cells were, in any case, not stimulated upon mobilization of intracellular Ca^{2+} by external ATP (Brown et al., 1995).

The identity of the whole cell Cl^- currents identified in this work requires further clarification. CFTR mRNA has been identified in rat primary inner medullary cell cultures (Husted et al., 1995). In addition, Morales et al. (1994) have mapped the expression of CFTR mRNA, using reverse transcription and amplification of cDNA using the polymerase chain reaction, along the rat nephron. CFTR is expressed in both the cortical collecting duct and the inner medullary collecting duct. Most interestingly, in addition to full-length CFTR a smaller product, perhaps representing a splice variant of CFTR, was present and most abundantly expressed in the medulla including in the IMCD (Schwiebert et al., 1994). When expressed in *Xenopus* oocytes the cRNA for the truncated form of CFTR gave cAMP stimulated currents which were reduced markedly compared to wild-type CFTR (Morales et al., 1994). In rat primary cultures of rat IMCD cells whole cell Cl^- currents stimulated by cAMP are correlated with the expression of CFTR mRNA detected after reverse transcription and PCR amplification (Husted et al., 1995).

Paulmichl et al. (1992) have identified a totally distinct protein coding for a Cl^- channel or Cl^- channel regulator (pI_{Cln}) using expression cloning in *Xenopus* oocytes using mRNA from dog-kidney MDCK cells. Northern blot analysis using a cDNA probe of the rat homologue of pI_{Cln} shows marked expression in rat renal medulla (Ishibashi et al., 1993). pI_{Cln} expression results in outwardly rectifying Cl^- currents blocked by both DIDS and NPPB at μM affinities (Paulmichl et al., 1992), while extracellular nucleotides, including ATP and cAMP, are also effective blockers. In addition to extracellular ATP having no effect on OR and linear currents, extracellular cAMP is also without effect on Cl^- current in mIMCD-3 cells (*not shown*). It is therefore unlikely that the whole cell currents in mIMCD-3 cells are mediated by pI_{Cln}. Recently, kidney-specific forms of the ClC family of Cl channels have been identified by molecular cloning techniques. Electrophysiological analysis of ClC-K1 expressed in *Xenopus* oocytes shows an outwardly rectifying Cl^- current sensitive to blockade by DIDS at 100 μM with an anion selectivity of $Br^- > Cl^- > I^-$ (Uchida et al., 1992). Immunohistochemical localization of ClC-K1 expression showed specific expression in the thin limb of Henles loop in the inner medulla (Uchida et al., 1995). cDNA encoding the homologous rat renal Cl^- channels ClC-K2L and ClC-K2S have also been recently identified (Adachi et al., 1994). Using microdissected nephron segments and Rt-PCR analysis of mRNA expression, it has been shown that ClC-K2 is expressed in segments where ClC-K1 is not; interestingly ClC-K2L mRNA is expressed in IMCD whereas ClC-K2S is less abundant (Adachi et al., 1994). Electrophysiological analysis of ClC-K2L/S expressed in *Xenopus* oocytes (Adachi et al., 1994) show similar characteristics namely, an outwardly rectifying Cl^- current showing time- and voltage-independence with an anion selectivity of $Br^- > I^- > Cl^-$ similar to the outwardly rectifying whole cell Cl^- current described in the present paper. However ClC-K2L Cl^- currents were inhibited by 60% in the presence of 100 μM external DIDS while diphenylamine-2-carboxylate was ineffective (Adachi et al., 1994). The properties of the mouse homologue of rat ClC-K2L channel are unknown.

Physiological Role of Cl^- Currents in mIMCD-3 Cells

What is the physiological role of the Cl^- currents identified in this study in mIMCD-3 cells? Despite mIMCD-3 cells being clonal in nature the data are consistent with at least 2 cell types expressing distinct Cl^- (and possibly Na^+) currents. Thus the possibility exists that 2 physiological roles are underpinned by the whole cell Cl^- currents identified in the present work. If present at the apical membrane, the high basal levels of Cl^- conductance may mediate Cl^- secretion or Cl^- absorption depending on the electrochemical gradient for

Cl^- across the apical membrane (Simmons, 1993; Schwiebert et al., 1994). The presence of such Cl^- conductances at the apical membrane together with expression of a basolateral $Na^+ + K^+ + 2Cl^-$ cotransporter (Delpire et al., 1994) implies that Cl^- secretion, if present, would not be affected by cAMP (in contrast to that reported by both Husted et al. (1995) and Kizer et al. (1995)). The identification of two distinct ATP-sensitive K^+ channels at the apical surface of mIMCD-3 cells (Sanson et al., 1994) suggests that the whole cell Cl^- currents identified in the present work may function in KCl secretion. Further work in which cell-attached patch clamp recordings are made to define the location of the Cl^- channels underlying the whole-cell Cl^- currents are required. In addition, studies utilizing reconstituted epithelia will help to define the functional role of the two distinct Cl^- currents in IMCD-3 epithelia.

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