A VOLTAGE-DEPENDENT Ca^{2+} INFLUX PATHWAY REGULATES THE Ca^{2+}-DEPENDENT Cl^- CONDUCTANCE OF RENAL IMCD-3 CELLS

John E Linley [a,b], Stefan H Boese[c], Nicholas L Simmons [a] and Michael A Gray[a]

[a] Epithelial Research Group, Institute for Cell and Molecular Biosciences, Medical School, Newcastle University, NE2 4HH, UK [b] Present address: Institute of Membrane & Systems Biology, University of Leeds, LS2 9JT [c]Zoophysiology, Institute for Biochemistry & Biology, University of Potsdam, Karl-Liebknecht-Str. 24-25, D-14476 Potsdam, Germany.

Key Words: Kidney, collecting duct, patch clamp, calcium-dependent chloride current, whole cell recording, Ca^{2+} influx.

Address correspondence to:

Michael Gray, Ph.D.
Epithelial Research Group
Institute for Cell and Molecular Biosciences
University Medical School
Newcastle University
NE2 4HH
UK

Tel: (+44) 0191 222 7592
Fax: (+44) 0191 222 7424
Email: m.a.gray@ncl.ac.uk

Abstract:

We have previously shown that the membrane conductance of mIMCD-3 cells at a membrane holding potential of 0 mV is dominated by a Ca^{2+} -dependent Cl^- current (I_{CLCA}). Here we report that I_{CLCA} activity is also voltage-dependent and that this dependence on voltage is linked to the opening of a novel Al^{3+}-sensitive, voltage-dependent, Ca^{2+} influx pathway. Using whole cell patch clamp recordings at a physiological holding potential (~60 mV) I_{CLCA} was found to be inactive and resting currents were predominantly K^+ selective. However, membrane depolarisation to 0 mV resulted in a slow, sigmoidal, activation of I_{CLCA} ($T_{0.5}$ ~ 500s) while repolarisation in turn resulted in a mono-exponential decay in I_{CLCA} ($T_{0.5}$ ~ 100s). The activation of I_{CLCA} by depolarisation was reduced by lowering extracellular Ca^{2+} and completely inhibited by buffering cytosolic Ca^{2+} with EGTA, suggesting a role for Ca^{2+} influx in the activation of I_{CLCA}. However, raising bulk cytosolic Ca^{2+} at -60 mV did not produce sustained I_{CLCA} activity. Therefore I_{CLCA} is dependent on both an increase in intracellular Ca^{2+} and depolarisation to be active. We further show that membrane depolarisation is coupled to opening of a Ca^{2+}-influx pathway that displays equal permeability to Ca^{2+} and Ba^{2+} ions and which is blocked by extracellular Al^{3+} and La^{2+}. Furthermore, Al^{3+} completely and reversibly inhibited depolarisation-induced activation of I_{CLCA} thereby directly linking Ca^{2+} influx to activation of I_{CLCA}. We speculate that during sustained membrane depolarisation, calcium influx activates I_{CLCA} which functions to modulate~~couple~~ NaCl transport across the apical membrane of IMCD cells.

INTRODUCTION

The renal inner medullary collecting duct (IMCD) is the final site of tubular filtrate modification and has the capacity for both net NaCl absorption and secretion depending on the prevailing physiological state [Rocha and Kudo, 1990, ~~21,~~ Wallace et al, 2002)~~27]~~. Using whole cell patch clamp current recordings at a holding potential of 0mV, we have previously shown that the dominant membrane conductance of mIMCD-3 cells (a model of the terminal portion of the IMCD), is due to an outwardly-rectifying, time–independent, calcium-dependent Cl^- current (I_{CLCA}) (Linley et al, 2007, ~~[14,~~Shindo et al, 1996, Stewart et al 2001~~ 24, 25)]~~. We have demonstrated that this conductance is acutely regulated by changes in extracellular calcium concentration as well as by increases in cytosolic Ca^{2+} brought about by $G_{q/11}$-coupled receptor agonists such as ATP, kinins and zinc ~~[~~(Linley et al, 2007, Stewart et al 2001)~~14, 25]~~. $G_{q/11}$-coupled receptor agonists also stimulate transepithelial Cl^- secretion in polarised monolayers under short circuit conditions ~~([~~Kose et al~~,~~ 2000)~~]~~. An interesting aspect of I_{CLCA} in mIMCD-3 cells is that the current does not display the intermediate time and voltage-dependent kinetic properties and rapid response to intracellular calcium seen for canonical calcium-activated Cl^- conductances in many epithelial cells, including some other collecting duct cell lines (Evans and Marty, 1986, Boese et al, 2000, 2004, Bertog et al, 1999~~Kormacher M1 cells??~~). Indeed, the unusually large magnitude of I_{CLCA} in mIMCD-3 cells measured under resting conditions at holding potentials of 0 mV, together with its regulation by external calcium levels, has suggested a working hypothesis in which a Ca^{2+}-influx pathway in mIMCD-3 cells is responsible for maintaining I_{CLCA} active by establishing a raised calcium level in a near-membrane domain. In collecting duct cells changes in intracellular calcium is an important determinant of duct cell function (Kose et al, 2000, Linley et al, 2007 ~~[12,) 14]~~ but the identity of the pathways responsible for calcium entry and homeostasis in the IMCD are poorly understood (Hoenderop et al, 2005~~[9~~, Magaldi et al 1989~~15)]~~.

There are a number of potential molecular candidates for the putative Ca^{2+} influx pathway in IMCD cells. The apical Ca^{2+} influx channels TRPV5/6 (Cat1/ECaC) are expressed in distal renal tubules; TRPV6 has a distribution along the nephron that continues past the DCT to include the medullary collecting duct ~~([~~Hoenderop et al, 2005~~9~~, Nijenhuis et al, 2003~~16)]~~. Polycystin-2 is a member of the TRPP sub-family, and localises to the plasma membrane and primary cilia of renal epithelia (Pazour et al~~[17~~, 2002, Yoder 2007~~28)]~~. Opening of polycystin-2 is linked to a flow-induced bending of the primary cilium and leads to calcium influx and Ca^{2+}-induced Ca^{2+}-release in renal cells (Zhao, 2002)~~[28]~~. Both L and T-type Ca^{2+} channels have also been shown to be expressed at the mRNA level in the IMCD (Andreasen et al, 2000, Zhao, 2002). ~~[1, 29]~~. Finally, alternative, less well characterised Ca^{2+} channels such as the mammalian homologue of the plant vacuolar Ca^{2+} channel AtTPC1, which shows high expression in rat IMCD, are potential candidates (Furuichi et al, 2001, Ishibashi et al, 2000, Peiter et al, 2005). In this report we provide evidence for a novel regulatory mechanism of I_{CLCA} in mIMCD-3 cells. We show that activation of I_{CLCA} required prolonged membrane depolarisation whereas hyperpolarisation reversed this process. The depolarisation-induced activation of I_{CLCA} was absolutely dependent on and preceded by Ca^{2+} influx through a voltage-dependent Ca^{2+} permeable pathway. This influx pathway was equally permeable to Ca^{2+} and Ba^{2+}, and was inhibited by Al^{3+} and La^{3+}, but not verapamil. These results therefore provide strong evidence to support our hypothesis that Ca^{2+} influx is required to maintain I_{CLCA} active in mIMCD-3 cells under depolarised conditions. The possible molecular nature of the Ca^{2+} influx pathway and its physiological relevance to Cl^- transport in IMCD cells are discussed.

Methods

Cell culture

mIMCD-3 cells (Rauchman et al, 1993,~~[20,~~ Vandewalle et al, 1999)~~ 26]~~ were grown in 75cm^2 Roux flasks without antibiotics in Hams F12 and DMEM (50/50v/v%) with 1 g/l glucose, 10% foetal calf serum and 2mM L-glutamine at 37^{0}C in humidified air:5% CO_2 (Shindo et al, 1996, Stewart et al 2001) ~~[24, 25]~~.

~~*M1 cells were cultured as above but with Hams F12:DMEM (50/50v/v%) with 4.5 g/l glucose, 5% foetal calf serum, 5μM dexamethasone with 100U/ml penicillin and 100μg/ml streptomycin. mIMCD-K2 cells were grown on Vitrogen 100 (purified collagen) coated flasks in OptiMem1 with Glutamax-I™ media supplemented with 10% fetal bovine serum and 50mg/l penicillin/streptomycin [2].~~~~ Formatted~~ For patch clamp experiments, mIMCD-3 were seeded on 24 mm glass coverslips in 6 well culture plates at a density of 2,200 - 11,000 cells/cm^2 and used 1 to 5 days later.

Patch clamp recording

Current recordings were mainly made using the perforated patch or “slow” whole cell recording technique and employed amphotericin B (240 μg/ml) or Nystatin (100-300 mg/ml) as the pore forming antibiotic. In some experiments fast whole cell recordings were made using conventional methods (Shindo et al, 1996) ~~[24]~~. Currents were amplified using an EPC-7 or EPC-9 patch clamp amplifier (HEKA Electronics, Lambrecht, Germany), filtered at 1KHz by an 8-pole Bessel filter then digitised at a sampling rate of 2KHz (EPC-7: CED 1401, U.K.; EPC-9: ITC-16, InstruTECH, USA). Steady state current/voltage (I/V) relationships were measured by applying 500 ms voltage pulses from V_{hold} to potentials between ±100 mV in 20 mV steps. The change in whole cell current with time was monitored by applying a 500 ms voltage pulse from -60 mV to +60 mV every 10 s, or from 0 mV to ± 60 mV, every 10 s. Series resistance and liquid junction potentials were corrected for as previously described (Stewart et al 2001)~~[25]~~. Whole cell currents

were calculated at the reversal potential (E_{rev}) ±60 mV, normalised to cell capacitance measured using the EPC-7/9 circuitry, and expressed as pA/pF, Relative ion permeabilities were calculated from the shift in E_{rev} upon changing bath ion concentration using the Hodgkin-Katz modification of the Goldman equation. In order to substantiate depolarization activation of an inward, Ca^{2+}-selective current, cell membrane current in perforated patch recordings were clamped to zero (in current clamp mode) and the resulting membrane potential (V_m) was measured. Replacement of the standard Na-rich bath solution (see below) with the standard pipette solution ($KCl_{in} = KCl_{out}$) plus 1 mM $CaCl_2$ at zero holding current should result in a membrane potential shift to ~ 0 mV if no Ca^{2+} conductance is present or activated. Rates of activation of I_{CLCA} by voltage were calculated from the slope of the linear phase of activation and expressed as the change in current density at +60 mV with time (pA/pF)/s.

Solutions & Chemicals

The standard bathing solution for patch clamp experiments contained (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgCl_2$ 1.2, NaH_2PO_4 0.3, KH_2PO_4 0.3, TRIS 14, Glucose 5 and was titrated to pH 7.4 with HCl. For ion selectivity experiments, 100 mM NaCl was replaced with an osmotic equivalent of mannitol, N-methyl-D-glucamine Cl, or KCl. The standard pipette solution contained (in mM) KCl 130, NaCl 10, $MgCl_2$ 2, Hepes 10, titrated to pH 7.4 with NaOH. Amphotericin/Nystatin was added from a DMSO stock to give a final concentration of 240 μg/ml or 100-300 mg/ml, respectively. For fast whole cell recording, TEA-Cl replaced KCl and 1mM ATP was present. In current clamp experiments the standard pipette solution was used to replace the standard bathing solution, except that total calcium was increased to 1.0 mM In experiments investigating divalent cation selectivity, all $CaCl_2$ was removed and replaced by the appropriate divalent (barium, manganese or strontium) Cl^- salt. For nominally calcium-free bath solutions, $CaCl_2$ was simply omitted. $AlCl_3$ was added directly to bath solutions prior to pH adjustment, but generation of HCl required additional base to bring this solution pH to 7.4.

Statistics

Data are expressed as the Mean ±SEM for n experiments. Statistical comparisons were performed using ANOVA with Bonferroni post-tests for multiple comparisons.

RESULTS

I_{CLCA} is regulated by membrane depolarisation.

Figure 1 demonstrates that I_{CLCA}, the large Ca^{2+}-dependent Cl^- conductance, of IMCD-3 cells [14, (Linley et al, 2007, Shindo et al, 1996, Stewart et al 2001)~~24, 25~~] is regulated by membrane voltage. When the cell was held mainly at a physiological membrane potential of -60 mV (with only brief excursions to +60 mV), steady state currents were small, outwardly-rectifying and moderately time-dependent at membrane potentials ≥80mV (Figure 1B(a)). Ion substitution experiments at –60mV, showed this basal conductance to have a high selectivity for K^+ over Cl^- (see supplementary Figure 1; mean shift in reversal potential when changing from a NaCl to KCl rich bath = +31.5 ± 5 mV, n = 8; mean shift in reversal potential when replacing 100mM bath NaCl with mannitol = +2.2 ± 5 mV, n = 5). In marked contrast when the membrane potential was held mainly at 0 mV, with only brief excursions to ±60 mV, there was a progressive increase in whole cell conductance following a delay/slow activation phase of ~60 s (Figure 1A). Under these conditions whole cell currents at +60 mV increased by approximately 6 fold, from 30.7 ± 3 (V_{hold} -60 mV) to 214.4 ± 22 (V_{hold} 0 mV) (paired t-test, n = 37, $p<0.001$), reaching a plateau after ~15 minutes. A similar increase in the current at -60mV was also observed (-18.4 ± 2 pA/pF at V_{hold} -60mV; -108.9 ± 9 pA/pF at V_{hold} 0mV). Whole cell currents under depolarised conditions were outwardly-rectifying but time-independent (Figure 1B(b)). The increase in whole cell conductance was accompanied by a shift in reversal potential (E_{rev}) towards the chloride equilibrium potential (from -26.4 ± 1mV to -12.7 ± 1mV) indicating that the activated conductance was now predominately Cl^- selective (paired t-test, n = 37, $p<0.001$). This was confirmed by replacement of bath NaCl (100mM) by mannitol which gave a shift in reversal potential of 17 ± 2 mV (n=10). The properties of this voltage-activated conductance are identical to those we have previously described for I_{CLCA} in mIMCD-3 cells (Shindo et al, 1996, Stewart et al 2001)~~[24, 25]~~. Therefore, membrane depolarisation to 0 mV was associated with a marked change in resting cell

permeability, with the cell membrane changing from being predominantly K^+ selective to predominantly Cl^- selective.

On return of the holding potential to -60 mV from 0 mV there was a relatively rapid decrease in I_{CLCA} to pre-stimulation levels indicating that the effect of membrane depolarisation was completely reversible (Figure 1A). However, an interesting feature of the response of I_{CLCA} to changes in membrane potential was the difference in kinetics between activation and inhibition; inhibition was well described by a mono-exponential decay with a $T_{0.5}$ of 90 ± 10 s (n=15) compared to activation that showed more complex kinetics. Here the activation process, after the shift to 0 mV, showed a sigmoidal type of response, with a slowly activating (lag)-period followed by an increase in current with a much longer $T_{0.5}$ (time to 50 % of plateau) of 472 ± 23 s (n=26). The difference in the activation/inhibition kinetics was maintained during multiple activation/inhibition cycles in the same cell (illustrated in Figure 5A).

The effect of holding potential on steady-state levels of I_{CLCA} was investigated further by monitoring the response of the cells to incremental depolarising steps from an initial holding potential of -80 mV. Stepwise depolarisations to test potentials of -60 mV, -40 mV, -20 mV and 0 mV resulted in a progressive increase in the steady-state whole cell current (measured at ±60mV) (Figure 2) resulting in a significant rise in the whole cell current at +60mV when the cell was depolarised past -40mV (-80mV vs. -20mV, $p<0.01$; -80mV vs. 0mV, $p<0.001$, paired t-test).

These results suggested that membrane depolarisation was linked to a slow activation of I_{CLCA}, and we suspected this was due to the entry of Ca^{2+} through voltage-sensitive channels. To test for this an identical voltage protocol was utilised but Ca^{2+} in the external solution was reduced. Note that we could not eliminate Ca^{2+} from the perfusion solution (e.g. by adding EGTA), because this led to the activation of a large cation-selective conductance in these cells (Stewart et al 2001) ~~[25]~~. However, using a nominally Ca^{2+}-free bathing solution (Linley et al, 2007)~~[14]~~, changing the holding potential from -60 mV to 0 mV still resulted in activation of the I_{CLCA} (Figure 3A).

However, the rate of activation (2.8 mM Ca^{2+}, 0.22 ± 0.06 pA/pF/s; nominally Ca^{2+} free, 0.11 ± 0.01 pA/pF/s , un-paired t-test, n = 7, $p<0.05$) and the mean steady-state current were significantly less than controls conducted in the presence of normal calcium levels (2.8 mM Ca^{2+}, 232.0 ± 29 & -132.9 ± 18 pA/pF; nominally Ca^{2+} free, 138.5 ± 32 & -75.7 ± 15 pA/pF; un-paired t-test, n = 7, $p<0.05$, Fig. 3A). Most calcium channels are also permeable to barium ions (Bean, 1989)~~(ref).~~ Repeating experiments where calcium was completely replaced with barium produced identical results in term of the rate and extent of activation of I_{CLCA}, (control, 0.31 ± 0.12 pA/pF/s; Ba^{2+} replacement 0.34 ± 0.08 pA/pF/s, n = 3). These results provide further support that depolarisation leads to opening of calcium-selective channels in mIMCD-3 cells.

Comment [A1]: I don't have the steady state current data on hand. I'll have to dig it out from my old hard drive.

If calcium entry was occurring under the conditions employed in our experiments then we reasoned that increasing the buffering capacity of the mIMCD-3 cells should also eliminate activation of $I_{CLCA.}$ To achieve this we performed additional experiments using the standard or fast whole cell recording configuration, employing a pipette solution containing a high concentration of the diffusible Ca^{2+} chelator EGTA (5 mM) (Figure 3B). In this case the steady-state currents at a holding potential of -60 mV were small and the resulting I/V relationship linear with a current density of 10.2 ± 2 & -9.9 ± 2 pA/pF (n = 8) comparable to the currents recorded in the perforated patch experiments (see above). However, when the holding potential was then changed from –60 mV to 0 mV, no significant increase in current density was observed (n = 8). Note that this finding contrasts with our previous experiments using a lower pipette EGTA concentration (0.2 mM) in which holding the membrane potential at 0 mV did lead to substantial I_{CLCA} activity (Shindo et al, 1996)~~[24]~~.

While these results so far suggested that membrane potential was only indirectly leading to the activation of I_{CLCA}, we wanted to explore this issue further by changing both the holding potential and the intracellular Ca^{2+} concentration. Using the fast whole cell technique, the intracellular Ca^{2+} concentration was fixed and the membrane potential clamped to either -60 mV or

0 mV (Figure 4). With the membrane potential clamped predominantly at -60mV, and $[Ca^{2+}]_i$ buffered to either 10nM or 1μM, I_{CLCA} activated transiently upon achieving the whole cell configuration however, the current then rapidly inhibited reaching a steady state current level which displayed a linear IV relationship. Similar rundown was observed with the membrane potential held predominantly at 0mV with $[Ca^{2+}]_i$ fixed to 10nM (5mM EGTA), indicating that depolarisation alone was insufficient for sustained I_{CLCA} activity. However, when Ca^{2+} was elevated to 1μM in combination with a holding potential of 0mV, I_{CLCA} currents did not display significant rundown. Taken together these data demonstrate that both depolarisation and Ca^{2+} are required for sustained activation of I_{CLCA}.

Identity of the depolarisation-activated calcium influx pathway.

We used a pharmacological approach to try and identify the putative Ca^{2+} influx pathway in mIMCD-3 cells using I_{CLCA} as an intrinsic sensor of near membrane Ca^{2+}. To investigate the contribution of voltage-sensitive L-type Ca^{2+} channels, verapamil (10 μM) was included in the bath solution prior to the change in the holding potential from -60 mV to 0 mV. Despite the presence of verapamil, depolarisation was still accompanied by an increase in the whole cell current which reached a plateau of 173.3 ± 20 & -86.1 ± 6 pA/pF over a similar time course compared to controls (Supplementary Figure 2). The steady-state currents displayed kinetics identical to I_{CLCA} activated in the absence of drug and were not significantly different in magnitude to controls (150.4 ± 22 & -78.1 ± 16, un-paired t-test, n = 3, $p>0.05$). Verapamil at an elevated dose (32 μM) was also without effect on fully activated I_{CLCA} under depolarised conditions (88 ± 18 % of values at +60 mV, n = 3, P = ns). Furthermore, using Fura-2 loaded cells to monitor intracellular Ca^{2+} levels [14, 24], addition of the L-type Ca^{2+} channel agonist, Bay-K 8644 (1 μM) had no effect on bulk cytosolic Ca^{2+} levels despite these cells displaying a normal extracellular ATP-mediated increase in cytosolic Ca^{2+} (Supplementary Figure 2D). Interestingly, no increase in

bulk cytosolic Ca^{2+} was observed upon membrane depolarisation (induced by high bath KCl), data not shown), suggesting that any calcium entry into the cell is limited to a region close to the plasma membrane.

We next tested the effect of Al^{3+}, which has previously been shown to block calcium influx mediated by AtTPC1 when expressed in plant cells (Kawano et al, 2004, Lin et al, 2005)~~[11,13]~~. TPC1 is highly expressed in inner medullary collecting duct cells at the apical plasma membrane (Ishibashi et al, 2000), however there is controversy as to whether TPC1 encodes a plasma membrane Ca^{2+} channel in mammalian cells (see discussion). Figure 5 shows that Al^{3+} (1 mM) was without effect on basal whole cell currents at –60 mV, however, the presence of Al^{3+} completely abolished the activation of I_{CLCA} when the holding potential was switched to 0 mV. The effect of Al^{3+} was fully reversible on washout of the cation (with holding potential maintained at 0 mV). Of particular note in these experiments is that I_{CLCA} activated very quickly upon washout of Al^{3+} with a time constant $T_{0.5}$ of ~110 seconds, which contrasts markedly to the slow activation of I_{CLCA} in the absence of Al^{3+}. Furthermore, the kinetics of depolarisation-induced I_{CLCA} activation were not modified after Al^{3+} exposure and washout (Figure 5A), suggesting that the cellular processes regulating I_{CLCA} were not disrupted in any way by the trivalent cation. Finally, Al^{3+} failed to inhibit the pre-activated Cl^- conductance at 0 mV (Figure 5B). Similar inhibitory effects on I_{CLCA} activation were also obtained with 1 mM La^+, although at this concentration the effect was poorly reversible (data not shown), Taken together these data indicate that Al^{3+} prevents the depolarisation-induced activation of I_{CLCA} by inhibiting the calcium-influx pathway, and not by a direct blocking action of the trivalent cation on I_{CLCA} itself. To substantiate our findings we then sought direct electrophysiological evidence for a voltage-dependent Ca^{2+} influx pathway which was sensitive to Al^{3+}. To do this we used whole cell current clamp experiments. Under these conditions, membrane depolarisation was achieved by switching from the standard NaCl-rich bath

solution to a KCl-rich solution (where KCl_{in} = KCl_{out}) but which contained an inwardly directed Ca^{2+} gradient (1 mM $[Ca^{2+}]_o$). Under such conditions the equilibrium potential (E_x) for K^+, Cl^- and Na^+ is 0 mV, whereas E_{Ca} would be predicted to be positive. Figure 6 shows that when the external NaCl bathing solution was switched to the KCl-rich solution, there was an initial movement of the membrane potential towards 0 mV, followed, after a delay of 20-25 s, by a marked shift in V_m to +21.2 ± 4.5 mV (n = 8) ($p<0.001$ vs zero mV and control values). This two step shift in V_m is therefore consistent with the delayed activation of a voltage-sensitive, Ca^{2+} permeable conductance due to membrane depolarisation Crucially, perfusion with Al^{3+} had no effect on V_m under control conditions, but abolished the establishment of a positive V_m upon switching to the KCl-rich bath solution. (V_m under these conditions was not significantly different from zero, 0.6 ± 2.8 mV, $p>0.2$, n = 6). In addition, application of Al^{3+} *after* depolarisation-activation of the Ca^{2+} influx pathway caused V_m to return to ~ 0mV (V_m changed from +21.9. ± 4.1 mV to 1.8 ± 2.7 mV, n=5), indicating that once activated, the calcium-influx pathway is still sensitive to Al^{3+} block. The establishment of a positive V_m upon switching to the KCl rich bath solution could also be prevented by removing Ca^{2+} from the bath solution thereby eliminating the driving force for Ca^{2+} influx (V_m = 3.10 ± 1.6 mV, n = 6, $p<0.05$ vs. 1mM $[Ca^{2+}]_o$, data not shown). These results provide strong evidence that membrane depolarisation leads to a time-dependant activation of a Al^{3+}-sensitive, Ca^{2+}-permeable channel in mIMCD-3 cells. The permselectivity of this calcium influx pathway was further investigated by repeating current clamp experiments in which external Ca^{2+} was replaced with different divalent cations (Ba^{2+}, Mn^{2+} and Sr^{2+}). After KCl depolarisation V_m shifted to +22.6 ± 3.8 mV; 5.3 ± 1.9 mV and 6.5 ± 2.1 mV, respectively (n = 3-5). These results indicate that the channel is equally permeable to calcium and barium, but less permeable to manganese and strontium. Note that we were unable to isolate a calcium/barium-selective current under voltage-clamp conditions due to contamination from other resting currents.

DISCUSSION

The primary site of active renal transcellular calcium reabsorption is the distal tubule via TRPV5 (Hoenderop et al, 2005)[9]. The collecting tubule, including the medullary collecting duct, is thought not to be implicated in bulk active Ca^{2+} reabsorption due to the absence of calbindins in OMCD and IMCD. Therefore, it is likely that alternative physiological functions exist for calcium channels in these segments (Nijenhuis et al, 2003)[16]. However, it should be remembered that early studies in perfused rat IMCD did demonstrate net Ca^{2+} reabsorption (Magaldi et al, 1989),[15] raising the possibility that apical Ca^{2+} channels in IMCD may participate in the regulation of urinary Ca^{2+} levels.

Here we report that IMCD cells contain a depolarisation-activated Ca^{2+} entry pathway that is functionally linked to the activity of a previously characterised Ca^{2+}-dependent Cl^- conductance (I_{CLCA}) (Linley et al, 2007, Shindo et al, 1996, Stewart et al 2001)[14, 24, 25]. We show that at a holding potential of –60 mV, I_{CLCA} is essentially inactive, and the cell is predominately K^+-selective. However, the Cl^- conductance slowly activates as the cells membrane potential is depolarised to below -40 mV, and at 0 mV I_{CLCA} then becomes the dominant membrane conductance. Since activation of I_{CLCA} was abolished by buffering intracellular Ca^{2+} or by blocking Ca^{2+} influx with Al^{3+} and was also sensitive to extracellular Ca^{2+} removal, we conclude that depolarisation-induced Ca^{2+} influx is an essential step in the activation of I_{CLCA}.

What is the molecular identity of the Al^{3+} sensitive Ca^{2+} influx pathway?

The α1G- T-type calcium channel is expressed in the IMCD and in mIMCD-3 cells (Andreasen et al 2000)[1], and inactivates rapidly in response to depolarizing stimuli. Since the activity of the Ca^{2+} influx pathway in mIMCD-3 cells is maintained for tens of seconds (Fig 6) under prolonged depolarisation, it is unlikely that a T-type Ca^{2+} channel participates in the

response reported here. L-type Ca^{2+} channels are also known to be stimulated by depolarising membrane potentials, are inhibited by verapamil and have been shown to be present at the mRNA level in mIMCD-3 cells (Zhao et al 2002)~~[29]~~. Addition of verapamil, either applied acutely or during the activation process failed to inhibit the subsequent activation of I_{CLCA} by depolarisation. Therefore, it is unlikely that influx of Ca^{2+} is through L-type Ca^{2+} channels. This conclusion is supported by evidence from *in vitro* microperfused rat IMCD which showed no verapamil-sensitive Ca^{2+} influx (Magaldi et al, 1989)~~[15]~~. Polycystin-2 is a member of the TRPP sub-family, and localises to the plasma membrane and primary cilia of renal epithelia (Pazour et al, 2002~~[17~~, Yoder, 2007~~28)]~~. Polycystin-2 is equally permeable to Na^+ and K^+ but shows greater permeability to Ca^{2+} (Gonzales-Perrett et al, 2001)~~[8]~~ and is inhibited by Gd^{3+} and La^{3+} (Yoder, 2007) ~~[28]~~. Although I_{CLCA} activation was found to be irreversibly inhibited by La^{3+} (data not shown), polycystin-2 channels are constitutively active at negative membrane potentials, and thus their voltage-dependence makes it unlikely that they are responsible for regulating depolarisation-induced activation of I_{CLCA}. The epithelial apical Ca^{2+} influx pathways TRPV5/6 family (Cat1/ECaC) are expressed in renal tubules; TRPV6 has a distribution along the nephron that continues past the DCT to include the medullary collecting tubule (Hoenderop et al, 2005, Nijenhuis et al, 2003)~~[9, 16]~~. A key feature of these channels is that they are constitutively active at resting membrane potentials; indeed current/voltage relationships show they are activated at hyperpolarizing potentials. More recently, Goel et al (2007) have shown that TRPC3 and 6 are expressed in collecting duct principal cells. TRPC3 together with aquaporin 2 were present at the apical plasma membrane after stimulation with vasopressin. Importantly, this co-expression was also seen in mIMCD-3 cells and transepithelial calcium transport in polarised layers was increased by overexpression of TRPC3 or reduced by a dominant negative TRPC3 construct (Goel et al, 2007). Similar increases in Ca^{2+} transport were seen when monolayers were stimulated by diacylgycerol analogues and by ATP, but not by thapsigargin (Goel et al, 2007). However, with

all these candidate Ca^{2+} influx pathways, either the biophysical properties, the pharmacological sensitivity or the physiological activation profile fail to identify a Ca^{2+} channel whose activity could explain our data.

Intriguingly, a mammalian homologue of the novel vacuolar Ca^{2+} channel AtTPC1 is highly expressed in epithelial cells of the IMCD (Ishibashi et al, 2000)~~[10]~~. Multiple sequence alignment studies showed that the channel contains a well conserved voltage sensor in S4 which suggest that TPC1 is likely to be voltage-gated (Ishibashi et al, 2000)~~[10]~~, and in plant (*Arabidopsis thaliana)*, the vacuolar Ca^{2+} channel is specifically blocked by Al^{3+} (Kawano et al 2004, Lin et al, 2005)~~[11,13]~~. AtTPC1 may also mediate transport across the plasma membrane since depolarisation induced by sucrose/H^+ cotransport can activate Ca^{2+} entry and Ca^{2+} signalling cascades (Furuichi et al, 2001). [7]. However, it remains unclear whether TPC1 confers a plasma membrane Ca^{2+} channel in mammalian cells. Attempts to characterise the electrophysiological properties of TPC1 in expression systems have so far been unsuccessful with no measurable membrane current observed when exogenously expressed in either CHO-K1 cells or *Xenopus* oocytes (Ishibashi et al, 2000)~~[10]~~. Therefore, TPC1 remains only a tenuous candidate for the voltage dependent Ca^{2+}-influx pathway described in IMCD-3.

Surprisingly there have been very few reports of the effect of Al^{3+} on either cloned or native Ca^{2+} channels. Busselberg et al (1993) reported that the endogenous voltage-dependent Ca^{2+} channels of rat dorsal root ganglion neurons were sensitive to block by Al^{3+} in a manner consistent with open pore block. Bobkov et al (2005) also reported that a TRP-like Ca^{2+} channel in lobster olafactory neurons was inhibited by *intracellular* Al^{3+}, La^{3+} Gd^{3+}.

I_{CLCA} requires both membrane depolarisation and calcium influx for sustained activity

The complex activation profile of I_{CLCA} at 0 mV (fig. 1) implies that multiple steps are involved in regulating this conductance. Although we don't yet have a complete understanding of

the whole process we believe our results suggest the following. Depolarisation to 0 mV activates calcium entry into IMCD cells via an Al^{3+}-sensitive electrogenic pathway (fig 6) and this event leads to the subsequent activation of I_{CLCA}. Ca^{2+} entry is likely to be localised to a region close to the plasma membrane as we did not detect any increase in bulk $[Ca^{2+}]_i$ in Fura-2 experiments after K^+ deploarisation.

Blocking Ca^{2+} influx with Al^{3+} prevented activation of I_{CLCA} by depolarisation (Fig 5) confirming that depolarisation alone cannot activate I_{CLCA}. However, because Al^{3+} only prevented I_{CLCA} activation if present *before* membrane depolarisation, and the trivalent ion had no effect on the pre-activated conductance (Fig 5B), indicates that sustained Ca^{2+} influx is not required to maintain I_{CLCA} fully active under depolarised conditions. This conclusion is further supported by the current clamp experiments where Al^{3+} was able to block the Ca^{2+} influx pathway after activation by membrane depolarisation. These results also show that Al^{3+} is not a blocker of I_{CLCA} itself. On the other hand, simply raising cytosolic Ca^{2+} to 1μM (at a membrane potential of -60mV) was not sufficient to cause a sustained activation of I_{CLCA} (fig 4). This implies that both membrane depolarisation and Ca^{2+} influx are needed for sustained activity. Thus, the observed interplay between membrane voltage, Ca^{2+} influx and I_{CLCA} activity cannot be satisfactorily explained by the direct activation of I_{CLCA} via an initial voltage-dependent Ca^{2+} influx. In addition, because pre-exposure to external Al^{3+} followed by washout of Al^{3+} (at 0 mV) led to a shortening of the ' activation lag' phase and also to an increased rate of activation of I_{CLCA} at 0 mV (Fig 4), indicates that there are other (as yet unidentified) voltage-dependent processes which contribute to the sustained activity of I_{CLCA} (at 0mV + Al^{3+}). Clearly, further experiments are required to fully understand the relationship between voltage, calcium entry and activation of I_{CLCA}.

What is the molecular nature of I_{CLCA} in IMCD cells?

Our previous work on the calcium-activated chloride conductances of mouse IMCD-derived cell lines have emphasised the differences between the early and late IMCD. In mIMCD-K2 cells (a model of the early IMCD) the calcium-activated chloride conductance displays strong time dependent activation kinetics at moderate $[Ca^{2+}]_i$ and an ion permeability sequence of $I^->Br^->Cl^-$ (Boese 2000), similar to the recently cloned calcium activated chloride channel TMEM16A (Caputo et al 2008, Schroeder et al, 2008 and Yang et al, 2008). In contrast, the I_{CLCA} of mIMCD-3 cells displays no time dependent activation kinetics at any $[Ca^{2+}]_i$ levels. Interestingly a number a splice variants of TMEM16A exist, one of which (TMEM16A(0)) confers a calcium activated chloride conductance which has reduced calcium sensitivity and no time-dependent activation kinetics, similar to the endogenous I_{CLCA} in mIMCD-3 cells. TMEM16A is a member of a family of closely related proteins of unknown function, which could provide a possible reason for the apparent diversity in biophysical properties and regulation of calcium-activated Cl^- channels in different cell types.

In summary we provide evidence for a depolarisation-activated Ca^{2+} channel in mIMCD-3 cells that is coupled to the activation of I_{CLCA}. The molecular nature of the Ca^{2+} influx pathway remains unknown, however, we speculate that it may be TPC1 based on its inhibition by Al^{3+} (a known blocker of AtTPC1) and TPC1 protein expression in IMCD. Further work is required using siRNA knockdown of TPC1 mRNA to investigate this possibility. Our results suggest a novel mechanism by which Na^+ absorption may couple Ca^{2+} influx to the activation of a Cl^- channel via membrane depolarisation in IMCD-3 cells. It is pertinent to note that bradykinin, ATP and other hormonal and paracrine agents stimulate transient increases in cytosolic Ca^{2+} in mouse IMCD cells, as well as activate I_{CLCA}, indicating that multiple Ca^{2+} regulatory pathways co-exist in these duct cells (Kose et al, 2000, Stewart et al, 2001)~~[12, 25]~~. The existence of a range of signalling pathways with polymodal activation properties allows integration of multiple stimuli to downstream effectors. Physiologically, apical membrane depolarisation of inner medullary

collecting duct cells would occur when Na^+ absorption is stimulated in these cells and a parallel activation of a Cl^- conductance would be expected to modulate transcellular NaCl transport in the IMCD (Chang et al, 2005)~~[4]~~.

Acknowledgements

We wish to thank Kidney Research for a studentship to JEL. JEL and SHB contributed equally to this work.

REFERENCES

Formatted

~~[1]~~ Andreasen D, Jensen BL, Hansen PB, Kwon T-H, Nielsen S, Skott O (2000) The alpha(1G)-subunit of a voltage-dependent Ca^{2+} channel is localized in rat distal nephron and collecting duct. Am J Physiol Renal Physiol 279: F997-1005

Bean BP (1989) Classes of calcium channels in vertebrate cells. Ann. Rev. Physiol. 51:367-384

Bertog M, Letz B, Kong W, Steinhoff M, Higgins MA, Biefeld-Ackermann A, Bunnett EW, Korbmacher C (1999) J. Physiol 521:3-17

Bobkov YV, Ache BW (2005) Pharmacological properties and functional role of a TRP-related ion channel inlobster olfactory receptor neurons. J. Neurophysiol 93:13721380

Formatted

~~[2]~~ Boese S, Glanville M, Aziz O, Gray MA, Simmons NL (2000) Ca^{2+} and cAMP-activated Cl^- conductances mediate Cl^- secretion in a mouse renal IMCD cell-line. J. Physiol 523: 325-338

Boese S, Aziz O, Simmons NL, Gray MA (2004) Kinetics and regulation of a Ca^{2+} activated Cl^- conductance in mouse renal inner medullary collecting duct cells. Am. J. Renal Physiol. 286:F682-F692

Busselberg d, Platt B, Michael D, Carpenter DO, Haas HL (1994) Mammalian voltage-activated calcium channels are blocked by Pb^{2+}, Zn^{2+} and Al^{3+}. J. Neurophysiol. 712: 1491-1497

Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, Pfeffer U, Ravazzolo R, Zegarra-Moran O Galietta LJV (2008) TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science 322: 590-594

[3] Carr G, Simmons NL, Sayer JA (2006) Disruption of clc-5 leads to a redistribution of annexin A2 and promotes calcium crystal agglomeration in collecting duct epithelial cells. Cell Mol Life Sci 63: 367-377

[4] Chang CT, Bens M, Hummler E, Boulkroun S, Schild L, Teulon J, Rossier BC, Vandewalle A (2005) Vasopressin-stimulated CFTR Cl- currents are increased in the renal collecting duct cells of a mouse model of Liddle's syndrome. J Physiol 562: 271-284

[5] Churchill GC, Okada Y, Thomas JM, Genazzani AA, Patel S, Galione A (2002) NAADP mobilizes Ca^{2+} from reserve granules, lysosome related organelles, in sea urchin eggs. Cell 111: 703-708

[6] Dellis O, Dedos SG, Tovey SC, Taufiq-Ur-Rehman, Dubel SJ, Taylor CW (2006) Ca^{2+} entry through plasma membrane IP3 receptors. Science 313: 229-233

Evans MG, Marty A (1986) Calcium-dependent chloride currents in isolated cells from lacrimal glands J. Physiol. 378:437-460

Formatted

[7] Furuichi T, Cunningham KW, Muto S (2001) A putative two pore channel AtTPC1 mediates Ca^{2+} flux in Arabidopsis leaf cells. Plant Cell Physiol 42: 900-905

Goel M, Sinkins WG, Zuo CD, Hopfer U, Schilling WP (2007) Vasopressin-induced membrane trafficking of TRPC3 and AQ2 channels in cells of the rat renal collecting duct. Am.J.Renal Physiol. 293: F1476-F1478

[8] Gonzales-Perrett S, Kim K, Ibarra C, Damiano AE, Zotta E, Batelli M, Harris PC, Reisin IL, Arnaout MA, Cantiello HF (2001) Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease (ADPKD) is a calcium permeable nonselective cation channel. Proc. Natl. Acad. Sci USA 98: 790-792

[9] Hoenderop JG, Nilius B, Bindels RJ (2005) Calcium absorption across epithelia. Physiol Rev 85:373–422

[10] Ishibashi K, Suzuki M, Imai M. (2000) Molecular cloning of a novel form (two-repeat) protein related to voltage-gated sodium and calcium channels. Biochem Biophys Res Commun 270: 370-376

[11] Kawano T, Kadano T, Fumoto K, Lapeyrie F, Kuse M, Osobe M, Furuichi T, Muto S (2004) Aluminum as a specific inhibitor of plant TPC1 Ca^{2+} channels. Biochem Biophys Res Commun 324: 40-45

[12] Kose H, Boese SH, Glanville M, Gray MA, Brown CDA Simmons NL (2000) Bradykinin regulation of salt transport across mouse inner medullary collecting duct epithelium involves activation of a Ca^{2+}-dependent Cl^- conductance. Br J Pharmacol 131: 1689-99

[13] Lin C, Yu Y, Kadano T, Iwata M, Umemura K, Furuichi T, Kuse M, Isobe M, Yamamoto Y, Matsumoto H, Yoshizuka K, Kawano T (2005) Action of aluminum, novel TPC1-type channel inhibitor, against salicylate-induced and cold-shock-induced calcium influx in tobacco BY-2 cells. Biochem Biophys Res Commun 332: 823-830

[14] Linley JE, Simmons NL, Gray MA (2007) Extracellular zinc stimulates a calcium-activated chloride conductance through mobilisation of intracellular calcium in renal inner medullary collecting duct cells. Pflugers Arch 453: 487-495

[15] Magaldi AJ, van Baak AA, Rocha AS (1989) Calcium transport across rat inner medullary collecting duct perfused in vitro. Am J Physiol 257: F738-F745

[16] Nijenhuis T, Hoederop JG, van der Kemp AW, Bindels RJ (2003) Localization and regulation of the epithelial Ca^{2+} channel TRPV6 in the kidney. J Am Soc Nephrol 14: 2731-2740

[17] Pazour GJ, San Augustin JT, Follit JA, Rosenbaum JL, Witman GB (2002) Polycystin-2 localizes to kidney cilia and the ciliary level is elevated in orpk mice with polycystic kidney disease. Curr Biol 12: R378-380

[18] Peiter E, Maathuis FJ, Mills LN, Knight H, Pelloux J, Hetherington AM , Sanders D (2005) The vacuolar Ca^{2+} -activated channel TPC1 regulates germination and stomatal movement. Nature 434:404-408

[19] Pottosin II, Schonknecht G (2007) Vacuolar calcium channels. J Exp Bot 58: 1559-1569

[20] Rauchman MI, Nigam SK, Delpire E, Gullans SR (1993) An osmotically tolerant inner medullary collecting duct cell line from an SV40 transgenic mouse. Am J Physiol 265: F416-424

[21] Rocha AS, Kudo LH (1990) Factors governing sodium and chloride transport across the inner medullary collecting duct. Kidney Int 38: 654-66

Schroeder BC, Cheng T, Jan YN Jan LY (2008) Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. Cell 134:1019-1029

7

[22] Sayer JA, Carr G, Morgan EL, Kellett GL, Simmons NL (2006) Expression and sub-cellular distribution of the two-pore channel 1 (tpc1) in mouse renal collecting duct cells. Proc Physiol Soc 2: PC22

[23] Sayer J, Carr, G, Simmons NL (2004) Calcium phosphate and calcium oxalate crystal handling is dependent upon CLC-5 expression in mouse collecting duct cells. Biochim et Biophys Acta 1689: 83-90

[24] Shindo M, Simmons NL, Gray MA (1996) Characterization of whole cell chloride conductances in a mouse inner medullary collecting duct cell line mIMCD-3. J Membr Biol 149: 21-31

[25] Stewart GS, Glanville M, Aziz O, Simmons NL Gray MA (2001) Regulation of an outwardly rectifying chloride conductance in renal epithelial cells by external and internal calcium. J Membr Biol 180: 49-64

[26] Vandewalle A, Bens M, Duong van Huyen JP (1999) Immortalized kidney epithelial cells as tools for hormonally regulated ion transport studies. Curr Opin Nephrol Hypertens 8: 581-587

[27] Wallace DP, Christensen M, Reif G, Belibi F, Thrasher B, Herrell D, Grantham JJ (2002) Electrolyte and fluid secretion by cultured human inner medullary collecting duct cells. Am J Physiol Renal Physiol 283: F1337-1350

Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim W0S, Park SP, Lee J, LeeB, Kim B-M, Raouf R, Shin YK, Oh U (2008) TMEM16A confers receptor-activated calcium-dependent chloride conductance Nature 455: 1210-1215

[28] Yoder BK (2007) Role of primary cilia in the pathogenesis of polycystic kidney disease. J Am Soc Nephrol 18: 1381-1388

[29] Zhao PL, Wang XT, Zhang XM, Cebotaru V, Cebotaru L, Guo G, Morales M, Guggino SE (2002) Tubular and cellular localization of the cardiac L-type calcium channel in rat kidney. Kidney Int 61: 1393-406

Formatted

FIGURE LEGENDS

Figure 1. I_{CLCA} is activated by cell depolarisation and inhibited by hyperpolarisation in mIMCD-3 cells. (A) Using the perforated whole cell patch clamp technique, the current at +60 mV (●) and -60 mV (○) was plotted in response to changing V_{hold} (see insets). (B) Steady state current traces obtained by 500 ms voltage pulses from V_{hold} to ±100 mV in 20 mV steps taken at (a) V_{hold} -60 mV and (b) V_{hold} 0 mV.

Figure 2. Summary of the effect of membrane holding potential on the magnitude of whole cell currents in mIMCD-3 cells. Membrane potential (V_{hold}) was held predominantly at the indicated voltage and steady state current density measured at E_{rev} +60 mV. Data expressed as mean ± SEM, n = 6-11. Data at 0 and –20 mV significantly different from data at –80 mV, $p<0.001$ and $p<0.01$, respectively.

Figure 3. Activation of I_{CLCA} by depolarisation is Ca^{2+} dependent. Whole cell patch clamp recording from mIMCD-3 cells illustrating the Ca^{2+} dependence of the depolarisation induced activation of I_{CLCA}. (A) Perforated whole cell patch clamp recording. Current at +60 mV (●) and -60 mV (○) is plotted with a nominally Ca^{2+} free bathing solution in response to changing the membrane holding potential (V_{hold}) as indicated by the upper bar. (B) Conventional fast whole cell recording with EGTA (5 mM) introduced into the cell cytosol through the patch pipette. (C) Mean results from (A) are expressed as current density measured at E_{rev} + 60 mV (upper columns) and E_{rev} − 60 mV (lower columns). Significant difference between the groups at V_{hold} = 0 mV is indicated with * (un-paired t-test, n = 7, $p<0.05$). Shading of bars indicates bath Ca^{2+} concentration.

Figure 4. Voltage and Ca^{2+} sensitivity of I_{CLCA}. Using the fast whole cell patch clamp technique, $[Ca^{2+}]_i$ was buffered to either 10nM (A) or 1μM (B) and the current density at +60 mV plotted. Data represent mean ± sem (A, n = 13; B, n = 14). Note that both membrane depolarisation and elevated cytosolic Ca^{2+} were required to maintain I_{CLCA} activity.

Figure 5. Al^{3+} blocks the depolarisation-activated whole cell currents in mIMCD-3 cells. **(**A) Individual record of whole cell current (perforated patch) in an IMCD-3 cell. Membrane holding potential (V_{hold}) is indicated in the upper bars and current at ±60 mV is plotted. Superfusion with 1 mM Al^{3+} at V_{hold} -60 mV prior to the 2nd activation cycle blocked activation of I_{CLCA} on switching V_{hold} to 0 mV. Activation occurred with a minimal delay after wash of Al^{3+} (1 mM). Final activation cycle was unchanged in comparison with pre- Al^{3+} exposure.
(B) Mean values of whole cell current densities obtained using the protocol illustrated in (A). The voltage/blocker sequence was , holding potential V_{hold} -60 mV, activation at V_{hold} 0 mV (control), return to V_{hold} -60 mV (a), superfusion with 1 mM Al^{3+} at V_{hold} -60 mV (b), depolarisation of V_{hold} to 0 mV (1 mM Al^{3+} present) (c), wash out Al^{3+} at V_{hold} 0 mV (d), superfusion with 1 mM Al^{3+} at V_{hold} 0 mV (e), finally wash out Al^{3+} & return to V_{hold} -60 mV. Mean data from n = 3 experiments.

Figure 6. Al^{3+} blocks a depolarisation-activated Ca^{2+} conductance. (A) Individual record of cell membrane potential (V_m) at zero holding current measured in current clamp mode. After external perfusion with the standard NaCl-rich bath solution, perfusion was switched to a KCl rich bath solution and 1 mM $AlCl_3$ was applied as indicated (see Methods). (B) Mean values (n = 6-8) of cell V_m. Measurements of V_m were taken at time points indicated by numbers, 1 to 7, in (A).

Supplementary material

Supplementary Figure 1: Whole cell currents at a holding potential of -60 mV were small and predominantly K^+ selective. (A) Typical current trace obtained by application of 500 ms voltage steps from the holding potential to ±100 mV in 20 mV steps with a standard NaCl rich bathing solution. (B) Typical current-voltage relationships showing the effect of changing the bathing solution from NaCl to KCl rich. Mean shift in reversal potential was +31.5 ± 5 mV (n = 8) indicating that the current was predominantly K^+ selective.

Supplementary Figure 2: L-type Ca^{2+} channels are not involved in depolarisation induced activation of I_{CLCA}. (A) Whole cell recording from mIMCD-3 cell with current at +60 mV (●) and -60 mV (○) plotted as in Figure 1A. Verapamil (10 μM) was added to the bathing solution as indicated by the lower bar. (B) Mean steady state data obtained using the protocol shown in (A). No significant difference in the magnitude of I_{CLCA} were observed in the presence or absence of verapamil (un-paired t-test, $p>0.05$, n = 3). (C) Steady state current trace at a holding potential of 0 mV after activation in the presence of verapamil. (D) mIMCD-3 cells were loaded with fura-2 AM and the ratio fluorescence ratio at 340/380 nm plotted. Cells were bathed with a KCl rich bathing solution to depolarise the cell and drugs added to the bath solution as indicated by the greyscale bars (Bay K 8644, 1 μM; ATP, 100 μM).

Figure 1

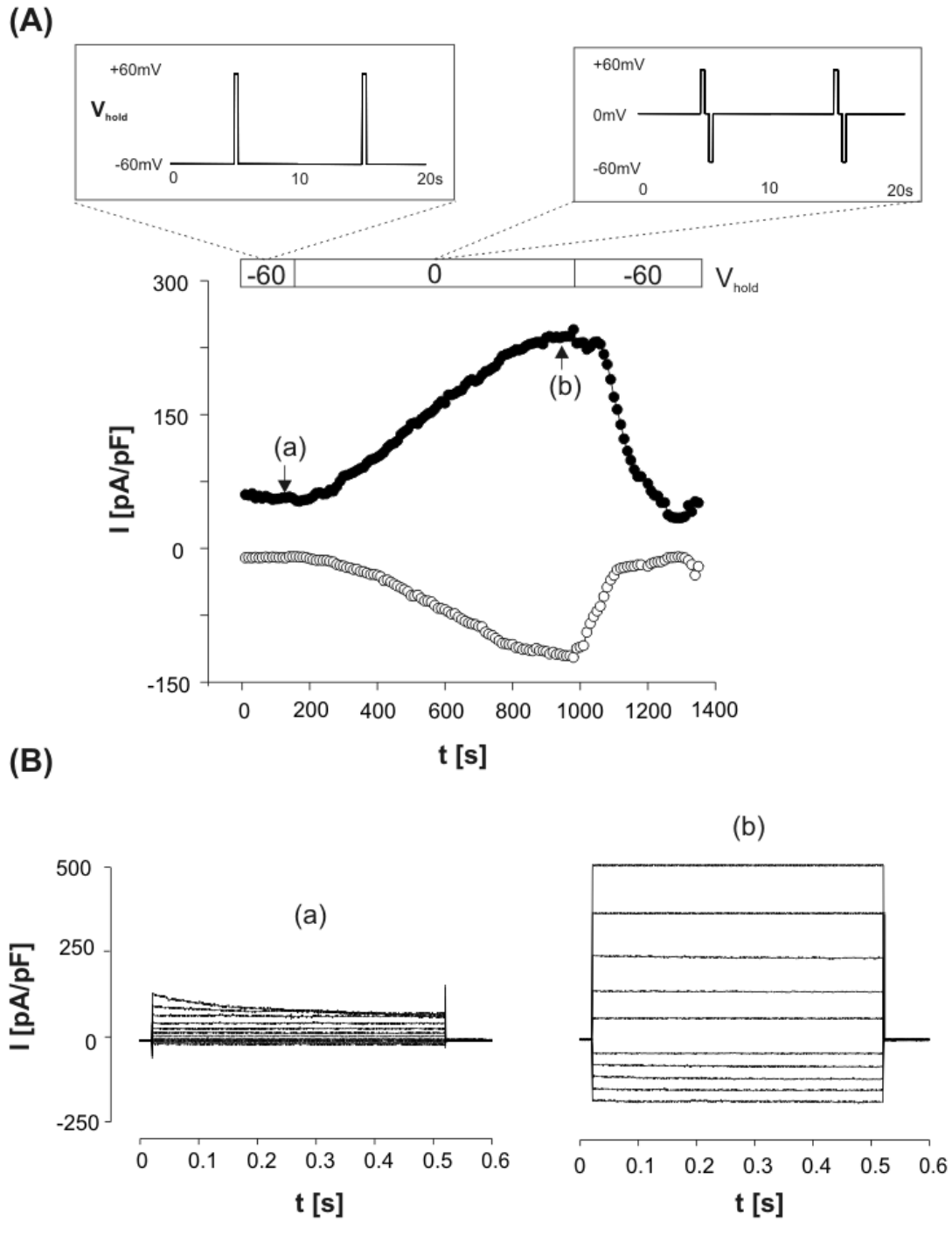

(A)
+60mV
V hold
-60mV
0
10
20s
+60mV
0mV
-60mV
0
10
20s
-60
0
-60
V hold
300
150
0
-150
I [pA/pF]
(a)
(b)
0
200
400
600
800
1000
1200
1400
t [s]
(B)
(a)
(b)
500
250
0
-250
I [pA/pF]
0
0.1
0.2
0.3
0.4
0.5
0.6
t [s]

Figure 2

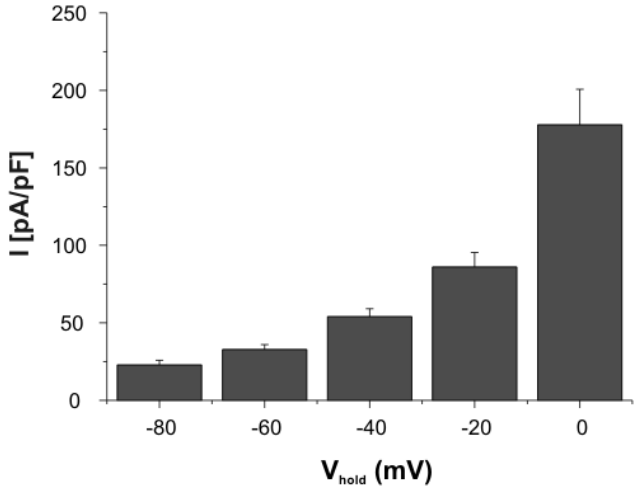


Figure 3

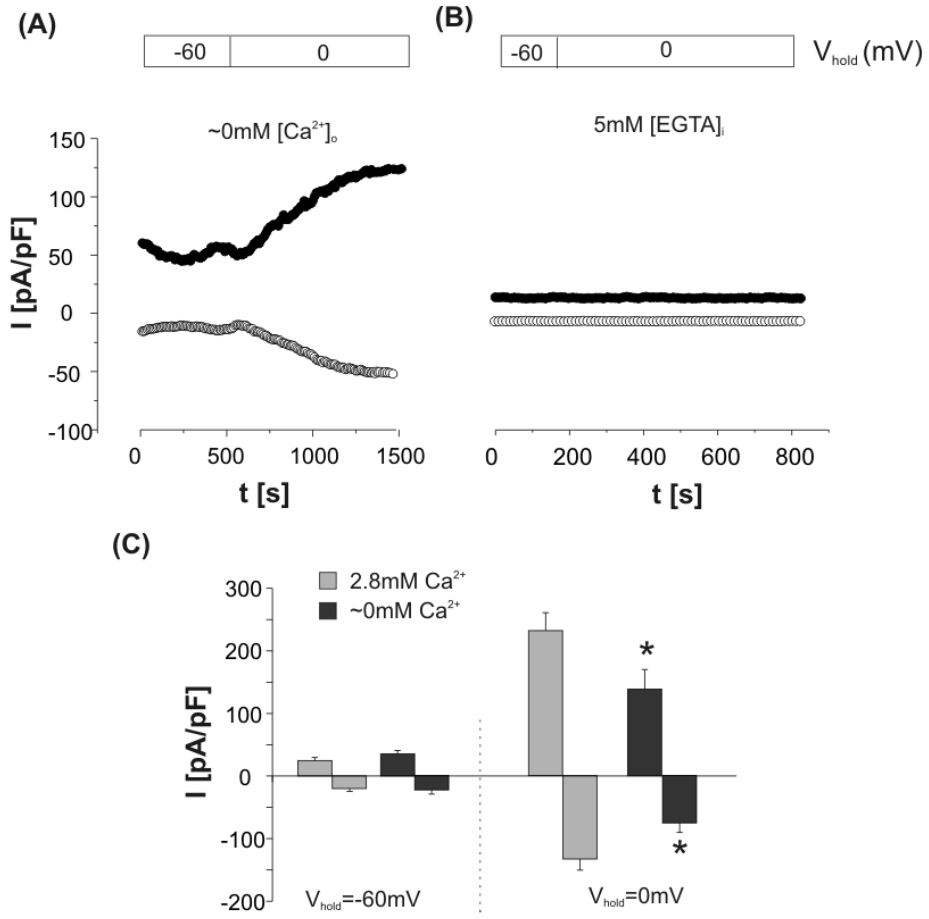

(A)
-60
0
~0mM [Ca2+]o
I [pA/pF]
150
100
50
0
-50
-100
0
500
1000
1500
t [s]
(B)
-60
0
Vhold (mV)
5mM [EGTA]i
0
200
400
600
800
t [s]
(C)
2.8mM Ca2+
~0mM Ca2+
I [pA/pF]
300
200
100
0
-100
-200
Vhold=-60mV
Vhold=0mV
*
*

Figure 4

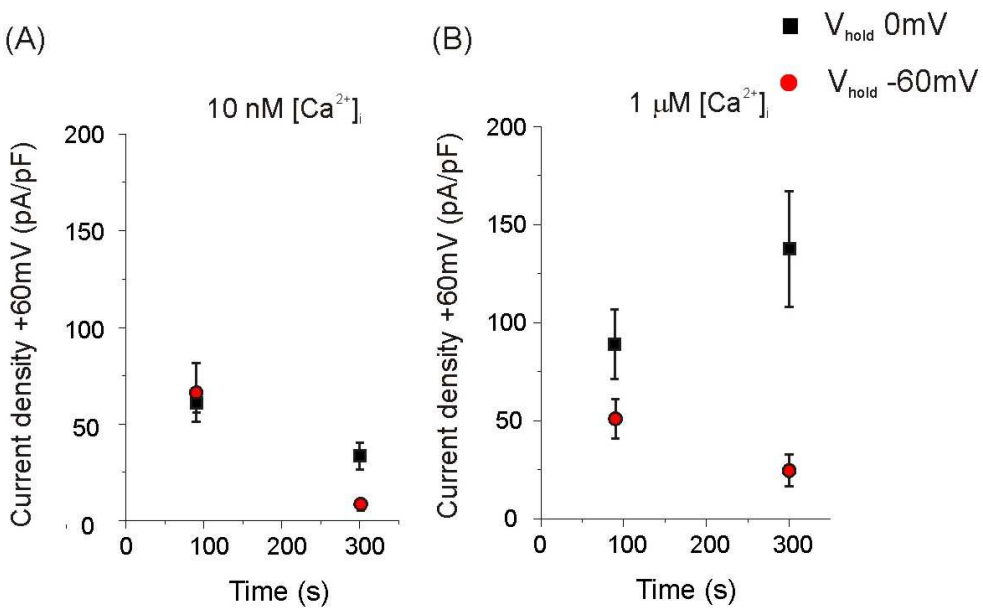

(A)
10 nM [Ca2+]i
Current density +60mV (pA/pF)
200
150
100
50
0
0
100
200
300
Time (s)
(B)
1 μM [Ca2+]i
Vhold 0mV
Vhold -60mV
Current density +60mV (pA/pF)
200
150
100
50
0
0
100
200
300
Time (s)

Figure 5A

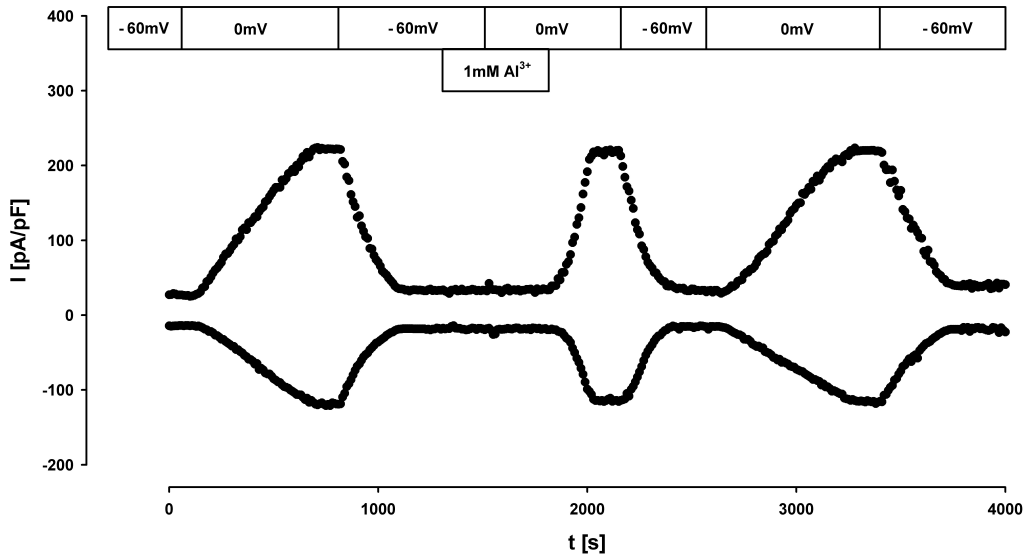
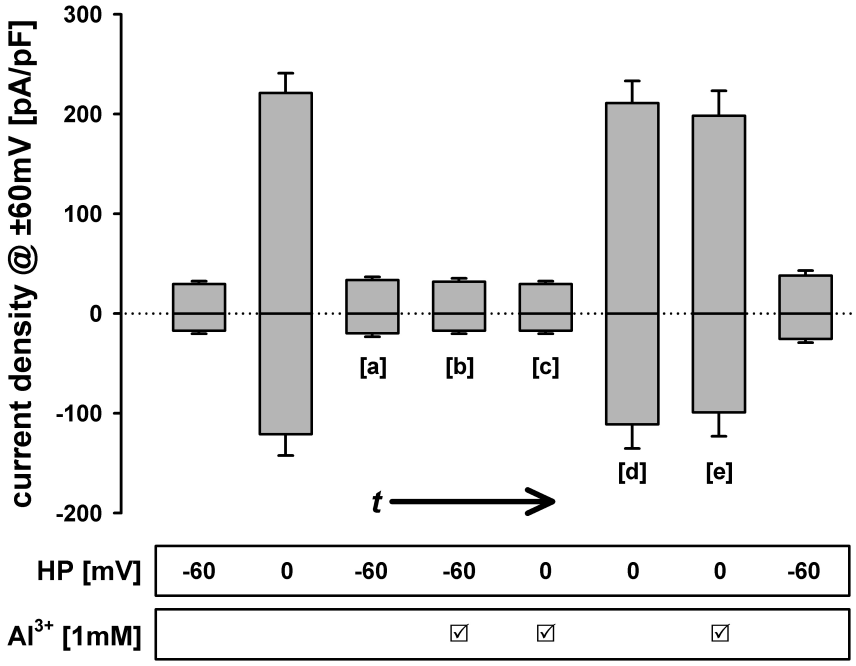

-60mV
0mV
-60mV
0mV
-60mV
0mV
-60mV
1mM Al3+
400
300
200
100
0
-100
-200
I [pA/pF]
0
1000
2000
3000
4000
t [s]

Figure 5B


current density @ ±60mV [pA/pF]
300
200
100
0
-100
-200
[a]
[b]
[c]
[d]
[e]
t
HP [mV]
-60
0
-60
-60
0
0
0
-60
Al3+ [1mM]
☑
☑
☑

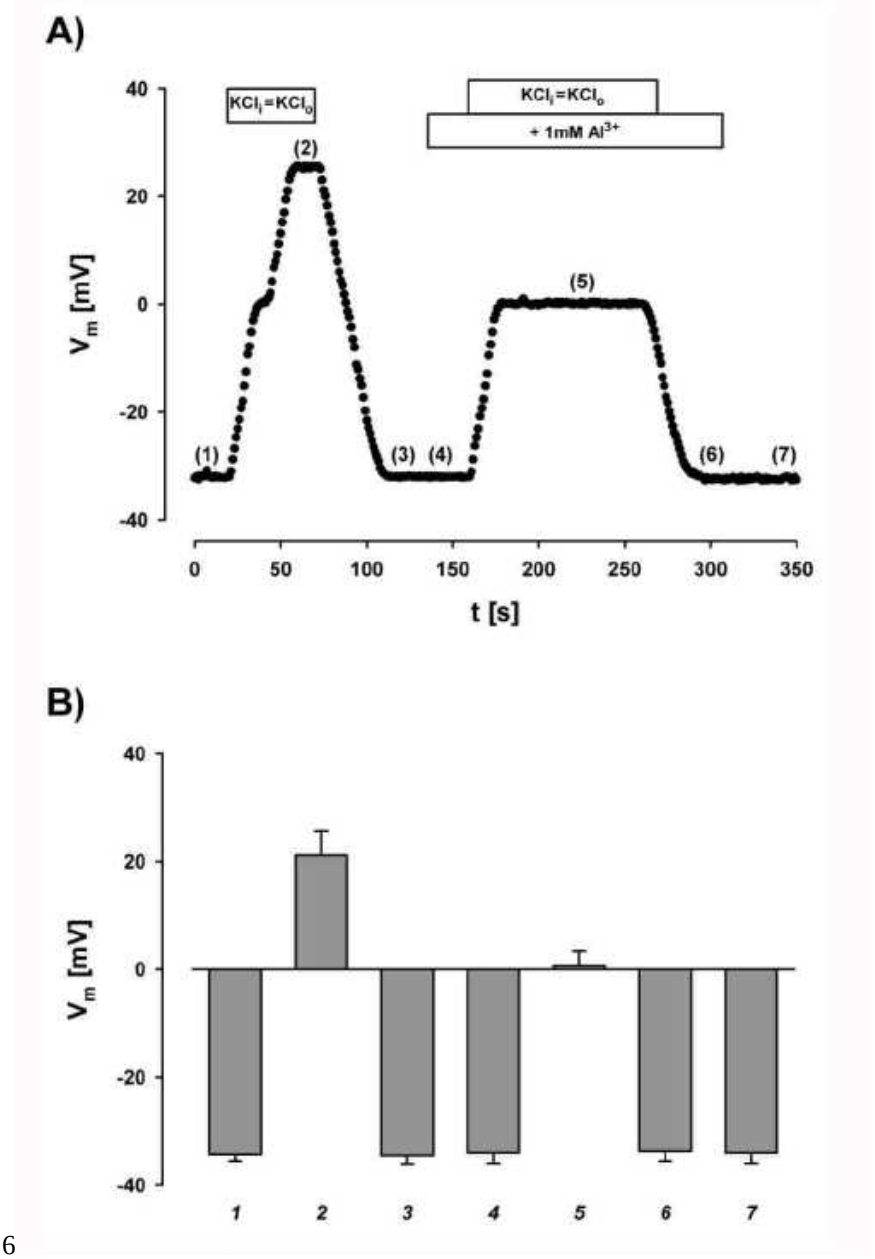
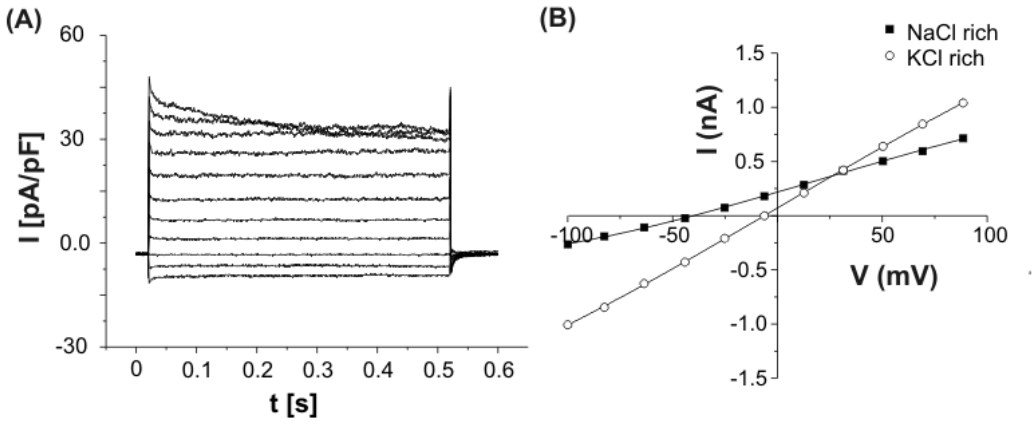


Figure 6

Supplementary figures

Supplementary Figure 1:



Supplementary Figure 2: