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Research article

NKCC2 activity is inhibited by the Bartter's syndrome type 5 gain-of-function CaR-A843E mutant in renal cells

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Background information. The gain-of-function A843E mutation of the calcium sensing receptor (CaR) causes Bartter syndrome type 5. Patients carrying this CaR variant show a remarkably reduced renal NaCl reabsorption in the thick ascending limb (TAL) of Henle's loop resulting in renal loss of NaCl in the absence of mutations in renal Na^+ and Cl^- ion transporters. The molecular mechanisms underlying this clinical phenotype are incompletely understood. We investigated, in human embryonic kidney 293 (HEK 293) cells and porcine kidney epithelial (LLC-PK1) cells, the functional cross-talk of CaR-A843E with the Na^+:K^+:2Cl^- co-transporter, NKCC2, which provides NaCl reabsorption in the TAL.

Results. The expression of the CaR mutant did not alter the apical localisation of NKCC2 in LLC-PK1 cells. However, the steady-state NKCC2 phosphorylation and activity were decreased in cells transfected with CaR-A843E compared with the control wild-type CaR (CaR WT)-transfected cells. Of note, low-Cl^--dependent NKCC2 activation was also strongly inhibited upon the expression of CaR-A843E mutant. The use of either P450 ω-hydroxylase (CYP4)- or phospholipase A2 (PLA2)-blockers suggests that this effect is likely mediated by arachidonic acid (AA) metabolites.

Conclusions. The data suggested that the activated CaR affects intracellular pathways modulating NKCC2 activity rather than NKCC2 intracellular trafficking in renal cells, and throw further light on the pathological role played by active CaR mutants in Bartter syndrome type 5.

Introduction

Bartter syndrome is a rare heterogeneous renal tubular disorder due to deficiency in Na^+ and Cl^- absorption. Clinical hallmarks are renal salt wasting, hypokalemic metabolic alkalosis, elevated serum renin and aldosterone levels with low to normal blood pressure. In some individuals, hypercalciuria is also present (Simon et al., 1996a; Simon et al.,1997; Konrad et al., 2000). Diverse subtypes of Bartter syndrome have been linked to mutations in one of the mechanisms involved in transepithelial NaCl transport across the distal nephron. In particular, loss-of-function mutations leading to Bartter syndrome have been described for transporters of the thick ascending limb (TAL) of Henle's loop such as the luminal bumetanide-sensitive Na^+-K^+-2Cl^- co-transporter 2 (NKCC2, Bartter syndrome type 1) (Simon et al., 1996a; Vargas-Poussou et al.,1998),

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Key words: Calcium homoeostasis/signalling, G-protein-coupled receptors, Intracellular signalling, Kidney, Lipid mediator.
Abbreviations: 20-HETE, 20-hydroxyeicosatetraenoic acid; 17-ODYA, 17-octadecynoic acid; AA, arachidonic acid; ADH, autosomal dominant hypocalcemia; CaR, calcium sensing receptor; ERK, extracellular signal-regulated kinases; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase; MDCK, Madin-Darby canine kidney; NKCC2, Na^+-K^+-2Cl^- co-transporter 2; pERK, phospho-ERK; PTH, parathyroid hormone; TAL, thick ascending limb.

the luminal potassium channel (ROMK, Bartter syndrome type 2) (Simon et al., 1996b) and the basolateral Cl^- channel (CLC-kb, Bartter syndrome type 3) (Simon et al.,1997; Konrad et al., 2000). Bartter's syndrome type 4 with sensorineural deafness has been linked to mutations of barttin, an accessory protein which works as an essential β-subunit for basolateral ClC-ka and ClC-kb channels (Estévez et al., 2001). Bartter syndrome type 5 is due to particular activating mutations of the extracellular calcium sensing receptor (CaR). The CaR enables key tissues involved in Ca^{2+} homoeostasis such as the parathyroid hormone (PTH)-secreting parathyroid glands, calcitonin-secreting thyroidal C cells, intestines, bones and kidney to constantly sense changes in extracellular Ca^{2+} levels (Brown and MacLeod, 2001). The CaR is a member of the G-protein-coupled receptor (GPCR) family and belongs to subfamily 3, which is characterised by a large amino-terminal extracellular domain. The CaR functions as a dimer, with dimerisation occurring through both covalent (intermolecular disulphide bonds) and noncovalent (hydrophobic) interactions between the extracellular domain monomers. The CaR signals via pertussis toxin-sensitive and -insensitive G proteins of the Gi and Gq/11 families respectively, to regulate second messengers that include cAMP, inositol triphosphate, diacylglycerol, intracellular Ca^{2+} and arachidonic acid (AA) metabolites (Hofer et al., 2000; Gerbino et al., 2005). These second messengers potentially regulate kinases, phosphatases and other signalling molecules. Like many GPCRs that act through Gi and Gq/11, the CaR stimulates mitogen-activated protein kinase (MAPK) signalling cascades, particularly the extracellular signal-regulated kinases (ERK) subfamily (Gamba and Friedman, 2009). Gain-of-function mutations in the CaR gene lead to the autosomal dominant hypocalcemia (ADH) disorder in which relative hypercalciuria often occurs (Pidasheva et al., 2004). In the kidney, CaR is mainly expressed on the basolateral membranes of cells of the medullary and cortical TAL (Chattopadhyay et al., 1996; Riccardi et al., 1998; Loupy et al., 2012; Toka et al., 2012). The pathogenetic mechanisms underlying Bartter syndrome type 5 have yet to be fully investigated. Of note, only some patients with ADH show an associated Bartter-like phenotype and thus far, explanations of the clinical phenotype have been based on the known physiological effects of extracellular Ca^{2+} in the TAL.

Under physiological conditions, activation of the CaR by increases in serum Ca^{2+} and Mg^{2+} concentrations inhibits divalent cation reabsorption in the renal tubule, which results in urinary loss of these mineral ions (Brown and MacLeod, 2001). This takes place primarily in the TAL, where divalent cation transport occurs via the paracellular pathway thought to be driven by the transepithelial voltage differences sustained by the NKCC2 co-transporter (de Rouffignac and Quamme, 1994). Inhibition of Ca^{2+} transport has been attributed to a selective inhibition of the paracellular permeability and to a reduction of the lumen-positive transepithelial voltage, secondary to a decrease in transcellular NaCl transport in this nephron segment (Brown and MacLeod, 2001).

One hypothesis to explain the Bartter syndrome type 5 is that activation of the CaR can inhibit directly or indirectly NKCC2, but the precise mechanism has not been investigated.

Here, we analysed the possible cross-talk between NKCC2 and the very active CaR-A843E mutant, responsible for a severe form of Bartter syndrome type 5 (Watanabe et al., 2002; Hendy et al., 2009; Hannan and Thakker, 2013).

Of note, unlike the majority of the other activating CaR mutants that only increase responsiveness to extracellular Ca^{2+} without affecting basal activity, CaR-A843E is constitutively active (Zhao et al., 1999). Substitution of glutamate for alanine 843 in the seventh transmembrane domain locks the receptor in an active conformation, disrupting the normal inhibitory constraints that prevent G protein coupling to the wild-type receptor in the absence of ligand.

We found that CaR-A843E strongly inhibits NKCC2 phosphorylation and activation without affecting NKCC2 trafficking in renal epithelial cells. This effect is independent of ROMK activity and likely involves AA metabolites.

Results

The A843E mutation constitutively activates the human CaR

Firstly, we evaluated real time CaR-stimulated changes in intracellular Ca^{2+} in single human embryonic kidney (HEK) 293 cells transiently transfected with human CaR WT or CaR-A843E, respectively. Cells loaded with Fura-2 AM were exposed to 2, 4 and

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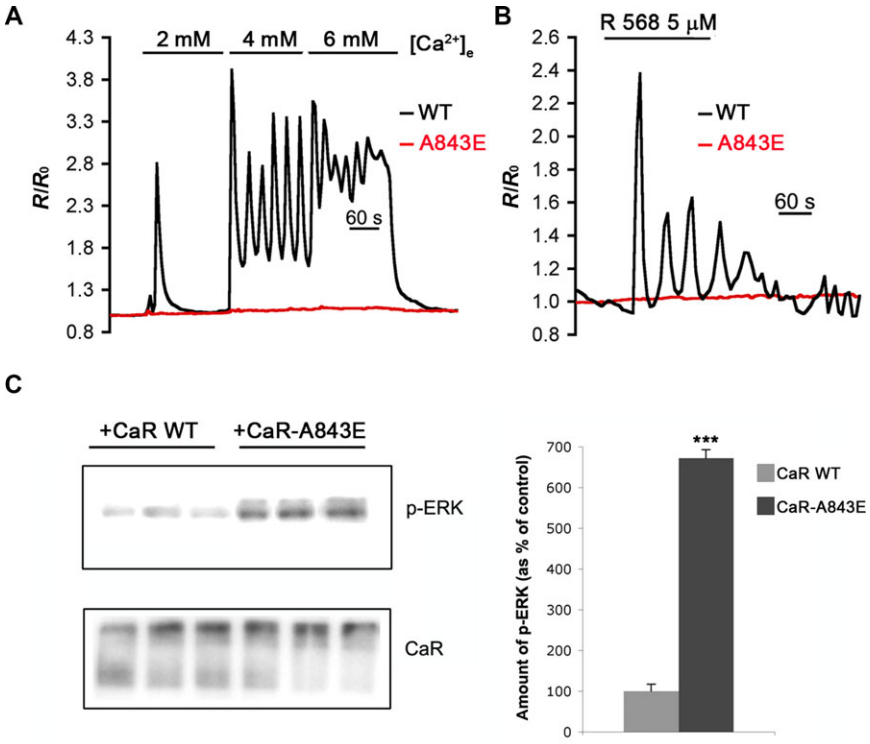
Figure 1 | CaR-A843E mutation constitutively activates the human CaR

Effects of either (**A**) increasing concentration of extracellular Ca^{2+} or (**B**) the calcymimetic R568 on intracellular Ca^{2+} levels. Cells transiently transfected with CaR WT (black trace) or CaR-A843E (red trace) were stimulated with either increasing levels of extracellular Ca^{2+} ($[Ca^{2+}]_{ext}$) or R568 and fluorescence ratio 340/380 nm was recorded and calculated as the change in fluorescence ratio (R) normalised to basal fluorescence ratio (R_0) observed in the absence of stimulus. (**C**) Phosphorylation of ERK1/2 is significantly increased in HEK 293 cells expressing the CaR-A843E mutant. Western blots were stained with antibodies against pERK and CaR as a loading control. ***$P < 0.001$, versus CaR WT.



6 mM extracellular Ca^{2+}, and variations in intracellular Ca^{2+} were evaluated by single-cell epifluorescence imaging and normalised with respect to basal levels (R_0) (Figure 1A). HEK 293 cells transfected with CaR WT had a threshold response to extracellular Ca^{2+} levels of about 2 mM and typical Ca^{2+} oscillations were observed in response to extracellular Ca^{2+} levels between 2 and 4 mM, whereas the maximal response, characterised by a sustained intracellular Ca^{2+} increase, occurred at 6 mM (Figure 1A; black trace, $n = 4$, $m = 51$ cells). In contrast, no change in fluorescence ratio was detected in cells expressing the CaR-A843E (Figure 1A; red trace, $n = 4$, $m = 38$ cells) and in mock-transfected cells ($n = 4$, $m = 70$ cells, data not shown). Secondly, for a more specific functional analysis of wild-type and mutant CaRs, the transfected HEK 293 cells were treated with R568, the positive allosteric CaR modulator that increases the sensitivity of the receptor to extracellular Ca^{2+} (Lu et al., 2009). R568 induced typical Ca^{2+} oscillations in cells expressing CaR WT (Figure 1B, black trace, $n = 4$, $m = 52$ cells), whereas no response was observed in CaR-A843E expressing cells (Figure 1B, red trace; $n = 4$, $m = 45$ cells). Unresponsiveness of CaR-A843 to both increasing extracellular Ca^{2+} levels and R568 is in agreement with the constitutive activity of the receptor and insensitivity to extracellular agonists shown in a phosphoinositide hydrolysis assay (Zhao et al., 1999; Watanabe et al., 2002). Thirdly, since the CaR also couples the MAPK

pathway (Brown and MacLeod, 2001), particularly the extracellular ERK subfamily (Tfelt-Hansen et al., 2003), we measured ERK 1/2 phosphorylation in HEK 293 cells expressing either the WT- or the mutant CaR. Basal phospho-ERK (pERK) activity was significantly and markedly greater in HEK 293 cells transfected with CaR-A843E when compared with those expressing CaR WT (Figure 1C), confirming the constitutive nature of the A843E mutation.

CaR-A843E inhibits NKCC2 phosphorylation and activity in NKCC2 transfected HEK 293 cells

To investigate the functional cross-talk between NKCC2 and CaR-A843E, we used HEK 293 cells stably transfected with the full-length NKCC2 (FL-NKCC2). Here, HEK 293 cells transfected with either CaR constructs showed diffuse membrane localisation of the CaR protein (Figure 2A, CaR-WT, CaR-A843E, red). Importantly, notwithstanding the exogenous expression of CaR WT and CaR-A843E, the transfected NKCC2 was properly expressed at the plasma membrane (Figure 2A, green) where it co-localised with the CaR (Figure 2A, merge, yellow), suggesting that the presence of the constitutively active CaR protein did not negatively affect the expression of NKCC2 at the membrane in these cells. To test whether CaR-A843E would affect NKCC2 activity, we performed the following NKCC activation assay. Intracellular Cl^- depletion activates NKCC co-transporters by promoting the phosphorylation of three highly conserved threonines (96, 101 and 111) in the amino terminus. The Cl^--sensitive activation of NKCCs requires its interaction with two serine-threonine kinases, WNK3 (with-no-lysine kinase) and SPAK (a Ste20-type kinase). WNK3 acts upstream of SPAK and appears to be the Cl^--sensitive kinase (Ponce-Coria et al., 2008). The activation assay was performed by incubating cells in a low Cl^- solution and analysing the phosphorylation state of the FL-NKCC2 by western blotting using the R5 antibody specifically raised against the conserved phospho-threonines in the amino-terminus of NKCCs. This analysis revealed a clear increase in the phosphorylation state of FL-NKCC2 in HEK 293 cells co-transfected with CaR WT in response to low Cl^- stimulation (Figure 2B). On the other hand, in cells co-transfected with CaR-A843E, the amount of phosphorylated FL-NKCC2 (p-NKCC2) was decreased both under basal conditions and upon low Cl^- activation relative to cells transfected with CaR WT (Figure 2B). Importantly, the CaR WT and CaR-A843E proteins were uniformly expressed in both experimental conditions. The phosphorylated FL-NKCC2 was significantly less well expressed by 60% and 120% in control and low Cl^- conditions, respectively, in cells transfected with CaR-A843E relative to those transfected with CaR WT (Figure 2C). Thus, expression of CaR-A843E was associated with a lower amount of phosphorylated FL-NKCC2.

CaR-A843E inhibits NKCC2 phosphorylation and activity in c-NKCC2 transfected LLC-PK1 cells

Next, we further studied the functional cross-talk between NKCC2 and CaR-A843E in the porcine kidney LLC-PK1 epithelial cell line. To overcome potential difficulties in stably expressing NKCC2 in renal epithelial cells, we had previously generated a functional chimeric NKCC2 protein (c-NKCC2) in which a 150 amino acid stretch of the NKCC2 C-terminal tail, containing the NKCC2 apical sorting determinants, were substituted in a NKCC1 backbone (Carmosino et al., 2008). The resulting c-NKCC2 is functionally active and accumulates at the apical membranes of stably transfected renal epithelial cells (Carmosino et al., 2008). Here, we transfected the c-NKCC2 stably expressing LLC-PK1 cell line with either CaR WT or CaR-A843E as appropriate. In each case, the CaR protein was correctly expressed at the plasma membrane of c-NKCC2-expressing LLC-PK1 cells (Figure 3A). Moreover, the expression of the CaR-A843E did not alter c-NKCC2 apical trafficking and expression (Figure 3A). To test whether CaR-A843E altered c-NKCC2 activity, the NKCC activation assay was conducted in LLC-PK1 cells. The phosphorylated c-NKCC2 was markedly decreased in the cells expressing the CaR-A843E mutant relative to those expressing WT CaR in both basal and low Cl^- conditions (Figure 3B). The CaR WT and CaR-A843E proteins were equivalently expressed under all experimental conditions. Although the phosphorylated c-NKCC2 was significantly increased in CaR WT-expressing cells upon low Cl^- stimulation, it was not increased, relative to baseline levels, in the cells expressing the CaR-A843E mutant (Figure 3C). Thus, the mutant CaR is associated with reduced phosphorylated c-NKCC2 under both basal and stimulated conditions.

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Figure 2 | CaR-A843E expression inhibits NKCC2 phosphorylation and activity in FL-NKCC2 transfected HEK293 cells (**A**) Confocal microscopy showing that the expression of CaR-A843E did not alter the localisation of NKCC2 in HEK 293 cells. (**B**) Western blotting analysis. CaR WT or CaR-A843E cell lysates were immunoblotted and probed with anti-p-NKCC2. (**C**) Densitometry of immunoreactive bands. Statistical analysis revealed that both p-NKCC2 expression and low Cl^--dependent activation of NKCC2, normalised against the CaR-transfected cells, were significantly lower in CaR-A843E transfected HEK 293 cells. $^{oo}P < 0.01$, low Cl^- versus control (CTR) in CaR WT-transfected cells. $^{**}P < 0.01$, CaR WT versus CaR-A843E under CTR conditions. $^{***}P < 0.001$, CaR WT versus CaR-A843E under low Cl^- conditions.

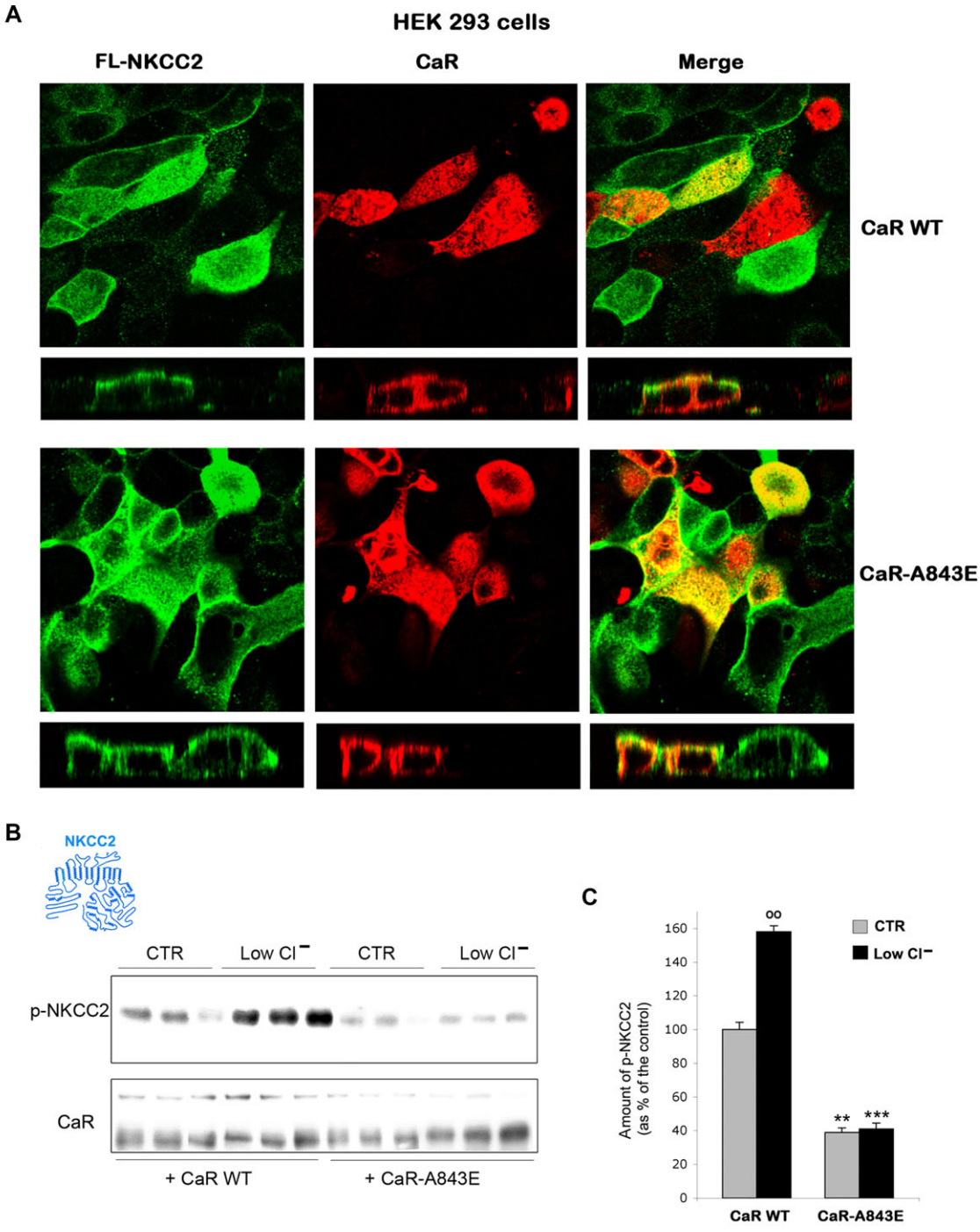
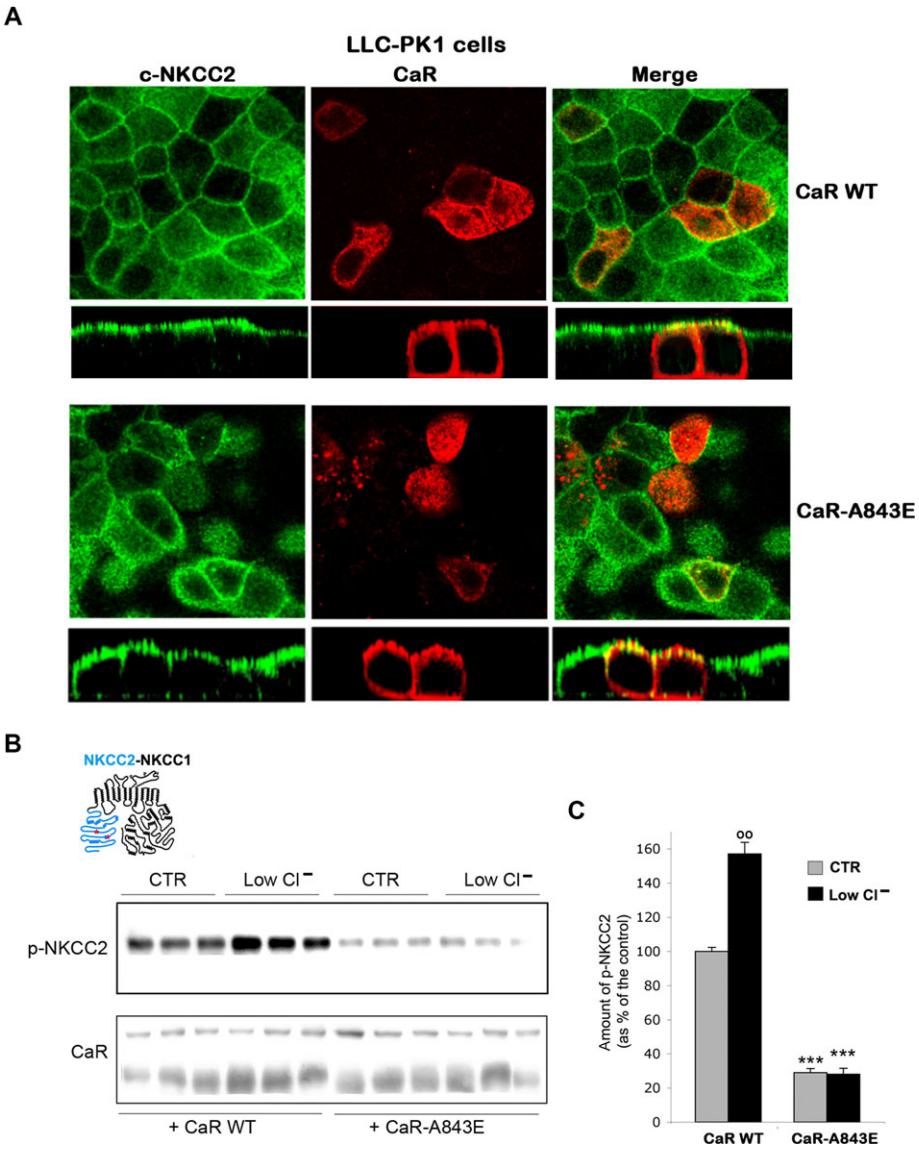


Figure 3 | CaR-A843E expression inhibits NKCC2 phosphorylation and activity in c-NKCC2 transfected LLC-PK1 cells (**A**) Confocal microscopy showing that the expression of CaR-A843E did not alter the apical localisation of c-NKCC2 in LLC-PK1 cells. (**B**) Western blotting analysis. CaR WT or CaR-A843E cell lysates were immunoblotted and probed with anti-p-NKCC2. (**C**) Densitometry of immunoreactive bands. Statistical analysis revealed that both p-NKCC2 expression and low Cl^--dependent activation of c-NKCC2, normalised against the CaR protein, were significantly lower in LLC-PK1 cells expressing the activating CaR mutant. $^{oo}P < 0.01$, low Cl^- versus CTR in CaR WT-transfected cells. $^{***}P < 0.001$, CaR WT versus CaR-A843E under CTR conditions. $^{***}P < 0.001$, CaR WT versus CaR-A843E under low Cl^- conditions.



CaR-A843E mimics the reduction in phosphorylated NKCC2 induced by activation of CaR

We hypothesised that the decreased phosphorylated NKCC2 levels associated with CaR-A843E could be attributed to the constitutive activation state of the mutant. Therefore, CaR WT ligand activation should mimic CaR-A843E expression in the absence of ligand, leading to decreased levels of phosphorylated NKCC2. In CaR WT-transfected LLC-PK1

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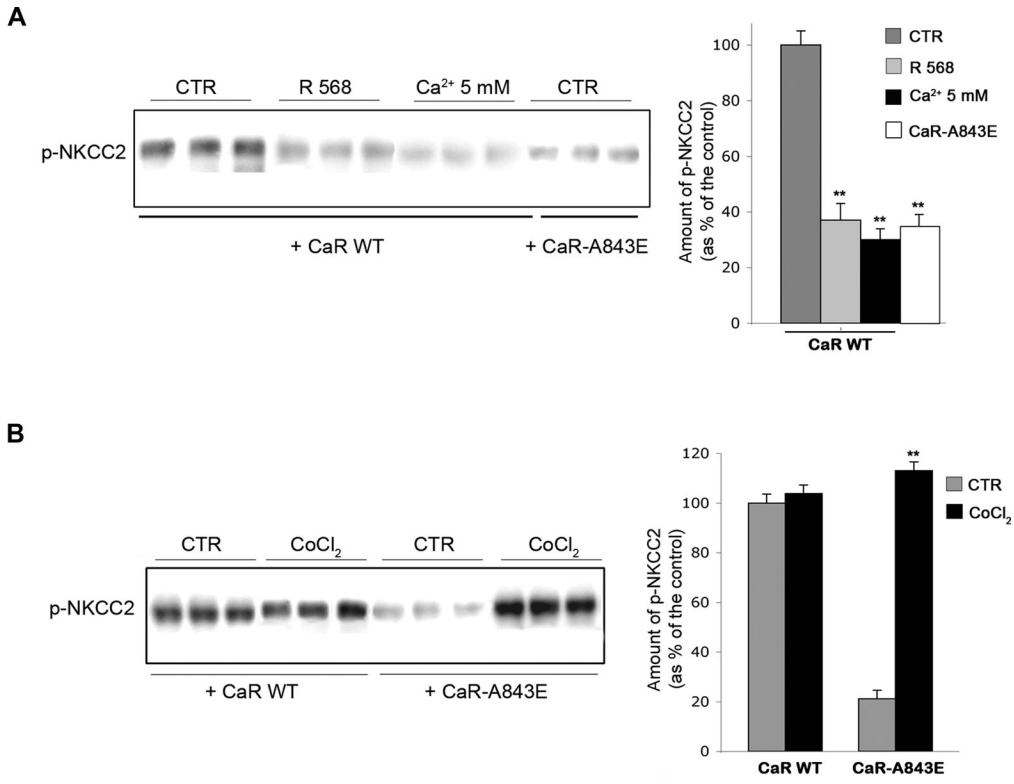
Figure 4 | CaR-A843E expression mimics the decrease in p-NKCC2 induced by CaR WT activation in LLC-PK1
(**A**) Left panel: phosphorylation of c-NKCC2 in LLC-PK1 cells expressing either CaR WT or CaR-A843E mutant, in low Cl^- buffer. Western blots were probed with antibodies against p-NKCC2. Right panel: densitometry of immunoreactive bands. Statistical analysis revealed that CaR-A843E expression mimics the inhibition of p-NKCC2 induced by CaR WT activation in LLC-PK1 cells. $^{**}P < 0.01$ versus CTR. (**B**) Left panel: phosphorylation of c-NKCC2 in LLC-PK1 cells expressing either WT- or mutant CaR, in low Cl^- buffer. Western blots were probed with antibodies against p-NKCC2. Right panel: densitometry of immunoreactive bands. Statistical analysis revealed that inhibition of CYP4 by $CoCl_2$ increases c-NKCC2 phosphorylation in LLC-PK1 cells transfected with CaR-A843E. $^{**}P < 0.01$ versus CTR in CaR-A843E-transfected cells.



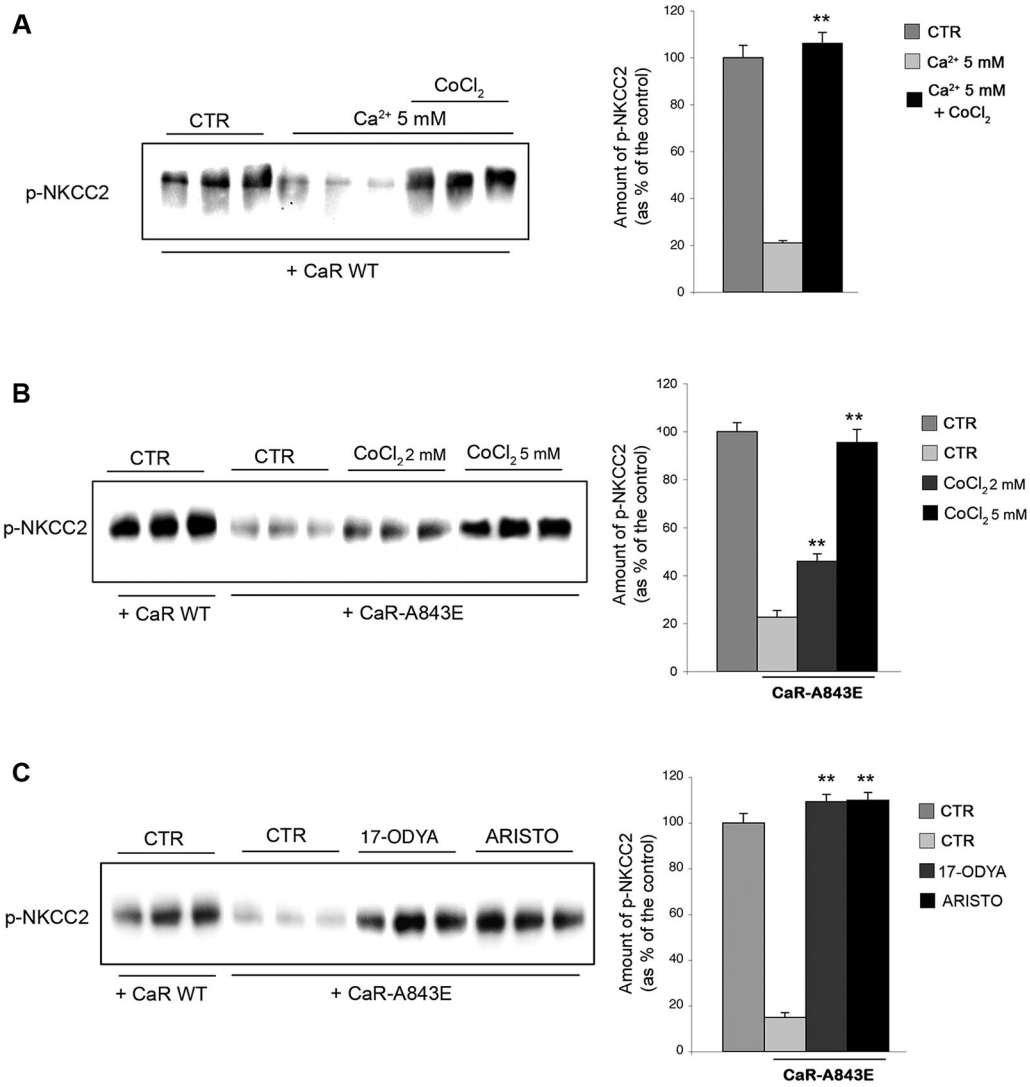
cells stimulated with either the calcimimetic R568 (1 μM) or high extracellular Ca^{2+} (5 mM), the levels of phosphorylated c-NKCC2 were reduced by 60% and 70%, respectively, relative to unstimulated control cells (Figure 4A). The reduced levels were similar to those observed in LLC-PK1 cells transfected with CaR-A843E, but in the absence of CaR agonists (Figure 4A).

The CaR signals through G proteins including those of the Gq/11 family that activate PLA2 (Brown and MacLeod, 2001). Of note, CaR-mediated activation of PLA2 in mouse TAL increases cytosolic AA, which is rapidly metabolised by a P450 ω-hydroxylase (CYP4) to 20-hydroxyeicosatetraenoic acid (20-HETE) (Wang et al., 2000). 20-HETE potently inhibits NKCC2 activity (Amlal et al., 1996). Thus, we performed the phosphorylation assay using the CYP4 inhibitor cobalt chloride ($CoCl_2$), which has been used to reduce CYP4 in many *in vivo* studies (Schwartzman et al., 1986; Escalante et al., 1991).

In LLC-PK1 cells expressing CaR-A843E treated with 5 mM $CoCl_2$, the inhibitory effect of the constitutively active mutant on c-NKCC2 phosphorylation was lost (Figure 4B). The levels of p-NKCC2 under those conditions were the same as those of cells expressing CaR WT treated either without or with 5 mM $CoCl_2$ (Figure 4B). Hence, the reduced activity of NKCC2 upon CaR activation is likely to be mediated by CYP4.

Figure 5 | Inhibitors of either CYP4 or aristolochic acid reversed the reduction of c-NKCC2 phosphorylation induced by expression of CaR-A843E in LLC-PK1 cells

(**A**) Left panel: phosphorylation of c-NKCC2 in LLC-PK1 cells expressing CaR WT, in low Cl^- buffer. Western blots were probed with antibodies against p-NKCC2. Right panel: densitometry of immunoreactive bands. Statistical analysis revealed that inhibition of CYP4 by $CoCl_2$ reversed the reduction of c-NKCC2 phosphorylation induced by 5 mM extracellular Ca^2 in LLC-PK1 cells transfected with CaR WT. $^{**}P < 0.01$ versus 5 mM Ca^{2+}. (**B**) Left panel: phosphorylation of c-NKCC2 in LLC-PK1 cells expressing either WT or A843E CaR in low Cl^- buffer. Western blots were probed with antibodies against p-NKCC2. Right panel: densitometry of immunoreactive bands. Statistical analysis revealed that the inhibitory effect of $CoCl_2$ on c-NKCC2 phosphorylation is dose-dependent. $^{**}P < 0.01$ versus CTR + CaR-A843R. (**C**) Left panel: phosphorylation of c-NKCC2 in LLC-PK1 cells expressing either WT or mutant CaR, in low Cl^- buffer. Western blots were probed with antibodies against p-NKCC2. Right panel: densitometry of immunoreactive bands. Statistical analysis revealed that inhibition of either CYP4 by 17-ODYA or PLA2 activity by aristolochic acid (ARISTO) reversed the reduction of c-NKCC2 phosphorylation induced by the expression of CaR-A843E in LLC-PK1 cells. $^{**}P < 0.01$ versus CTR + CaR-A843R.



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We performed several controls to corroborate our conclusions. Firstly, in LLC-PK1 cells expressing CaR WT treated with 5 mM $CoCl_2$, the inhibitory effect of 5 mM Ca^{2+}-activated CaR on c-NKCC2 phosphorylation was lost (Figure 5A), thus suggesting that the inhibitory effect of the CaR-associated pathway on c-NKCC2 activity is mediated by CYP4 also under physiological conditions.

Secondly, the effect of $CoCl_2$ was dose dependent. In LLC-PK1 cells expressing CaR-A843E treated with 2 mM $CoCl_2$, the level of phosphorylated c-NKCC2 was reduced relative to those treated with 5 mM $CoCl_2$ (Figure 5B). Moreover, 17-octadecynoic acid (17-ODYA), a selective inhibitor of CYP4 in the outer renal medullae (Stec et al., 1997), counteracted, at the same extent of 5 mM $CoCl_2$, the inhibitory effect of CaR-A843E on c-NKCC2 phosphorylation, thus suggesting that the renal CYP4 is specifically involved in NKCC2 inhibition upon CaR-A843E expression (Figure 5C). In addition, aristolochic acid, a known specific PLA2 inhibitor (Vishwanath et al., 1987; Lindahl and Tagesson, 1993), completely reverted the inhibitory effect of the constitutively active mutant on c-NKCC2 phosphorylation in LLC-PK1 cells expressing CaR-A843E (Figure 5C), suggesting that the CaR-A843E-induced reduction of the phosphorylated c-NKCC2 is likely dependent by PLA2 activation.

Discussion

In this study, we investigated the functional cross-talk between the constitutively active CaR-A843E mutant and NKCC2, the main transporter involved in NaCl reabsorption in the TAL.

Several mechanisms have been proposed to account for the inhibitory effect of the active CaR on paracellular divalent cations transport in the TAL.

In rat isolated TAL, by using the patch-clamp technique with fluorescent dyes, it was shown that CaR activation reduces the activity of the apical ROMK (Wang et al., 1997).

Because in the TAL K^+ secretion by ROMK is essential for maintaining the positive luminal potential that drives paracellular reabsorption of Ca^{2+} and Mg^{2+}, CaR-mediated inhibition of ROMK increases their urinary excretion. Moreover, inhibition of ROMK further limits the rate of apical Na^+-K^+-$2Cl^-$ co-transport, which requires K^+ recycling across the apical membrane, thus further reducing the positive luminal potential. In contrast, other studies have shown alterations in Ca^{2+} reabsorption without changes in transepithelial potential or NaCl transport (Motoyama and Friedman, 2002; Loupy et al., 2012). Recently, it has been shown that the extracellular Ca^{2+} concentration, mediated by the CaR, regulates the activity of the basolateral K^+ channel, suggesting another mechanism by which CaR modulates the reabsorption of divalent cations in the TAL (Kong et al., 2012).

In addition, activation of CaR reduces hormone-induced cAMP production probably through the activation of phospholipase C and a Ca^{2+}-inhibitable type-6 adenylyl cyclase expressed in cortical (c) TAL (de Jesus Ferreira et al., 1998). Therefore, CaR-dependent reduction of cAMP/PKA-mediated phosphorylation of NKCC2 and ROMK, a fundamental mechanism regulating the activity of both transporters (McNicholas et al., 1994; De Jesus Ferreira and Bailly, 1998), should also inhibit NaCl reabsorption and K^+ secretion in cTAL. Moreover, activation of CaR induces lysosomal degradation of claudin-16, one of the cation-permeable channels responsible for paracellular transport of Ca^{2+} and Mg^{2+} in Madin-Darby canine kidney (MDCK) cells (Ikari et al., 2008).

Interestingly, increases in the extracellular Ca^{2+} concentration inhibits Na^+-K^+-ATPase activity in the proximal tubule, potentially participating in the natriuretic effect of hypercalcemia (Levi et al., 1987), and suggesting that the Na^+-K^+-ATPase may be similarly inhibited in TAL cells upon CaR activation.

In addition to these multiple possible mechanisms, the present study suggests for the first time that CaR activation may results in direct inhibition of NKCC2 co-transporter in renal cells, thus providing insights into the direct cross-talk of CaR and NKCC2 under physiological conditions as well as the pathogenesis of Bartter syndrome type 5.

The CaR-A843E mutant that we utilised in our studies is of particular interest because A843E is unusual among other CaR ADH-causing mutations since being truly constitutively activating rather than causing an enhanced Ca^{2+} sensitivity (Zhao et al., 1999).

Accordingly, in our cellular models, we found that CaR-A843E mutant does not alter the level of intracellular Ca^{2+} transients under high extracellular Ca^{2+}

concentration relative to basal nor under treatment with a positive allosteric CaR modulator.

Interestingly, NKCC2 phosphorylation was markedly reduced in renal cells expressing either CaR-A843E mutant under basal conditions or the CaR WT under ligand-stimulated conditions.

The NKCC2 activation assay we used is based on inducing intracellular Cl^- depletion, which in turn activates the low Cl^--sensitive WNK3. This kinase then activates SPAK, which directly phosphorylates the three highly conserved threonines (96, 101 and 111) in the N- terminus of NKCCs (Ponce-Coria et al., 2008).

Since the assay was performed in a K^+-free Ringer's solution (see *Materials and Methods*), we can conclude that the active CaR directly targets the NKCC2 phosphorylation pathway, rather than either the ROMK, basolteral K^+ channel or Na^+-K^+-ATPase activities. Thus, CaR WT may directly talk with NKCC2 independently of the other K^+ transporters. Likewise, activation of CaR inhibited the phosphorylation of a chimeric NKCC construct containing the N-terminal part of NKCC1, with the well-conserved phosphoregulatory threonine residues and the C-terminal apical sorting determinant of NKCC2.

The chimeric c-NKCC2 protein exhibits proper apical expression and functional activity in epithelial cells (Carmosino et al., 2008, 2010, 2012).

The expression of CaR-A843E in LLC-PK1 cells did not alter the intracellular trafficking of c-NKCC2, but rather, targeted the activity of NKCC2.

Interestingly, the inhibitory effect of CaR-A843E on NKCC2 phosphorylation was abrogated in the presence of $CoCl_2$ and 17-ODYA, two potent CYP4 inhibitors (Escalante et al., 1991; Stec et al., 1997), suggesting the prostaglandin metabolite 20-HETE as the intracellular messenger linking CaR activity to NKCC2 inhibition. In addition, the possible involvement of PLA2 activation, upstream of the 20-HETE production, was suggested by experiments performed in the presence of a specific PLA2 blocker.

It was previously reported that 20-HETE inhibits NKCC2-mediated Na^+ and Cl^- transport in TAL tubules (Escalante et al., 1991; Escalante et al., 1994) but the molecular mechanism underlying this effect was not clear. Our studies suggest that 20-HETE may directly inhibit NKCC2 activity interfering with its phosphorylation pathways.

In conclusion, our findings shed light on the molecular mechanisms involved in the regulation of NKCC2 in the TAL upon CaR activation under physiological conditions, and provide insights into the pathogenesis of Bartter syndrome type 5 that may open new avenues to pharmacological interventions in this disorder.

Materials and methods

Constructs and antibodies

The chimeric c-NKCC2 and the full-length FL-NKCC2 correspond to the previously described chimera IV–VI and NKCC2, respectively (Carmosino et al., 2008). The c-Myc-tagged CaR WT was described previously (D'Souza-Li et al., 2001) and CaR-A843E mutant was prepared by site-direct mutagenesis. The correctness of the construct was confirmed by sequencing. The polyclonal R5 antibody against two regulatory phosphothreonines in the NKCC N-terminus was generated and characterised by Prof. Biff Forbush (Yale University). The monoclonal HA antibody was from Covance (www.covance.com) and the polyclonal anti-c-Myc antibody was from Sigma–Aldrich (www.sigmaaldrich.com). The polyclonal pERK 1/2 antibody was from Cell Signaling (www.cellsignal.com).

Cell culture and transfection

LLC-PK1 and HEK 293 cells were maintained in Dulbecco's modified Eagle's medium high glucose, 2 mM L-glutamine, 10% foetal bovine serum, penicillin (50 U/ml) and streptomycin (50 U/ml) at 37°C, 5% CO_2 in a humidified incubator. For transfection, cells were grown until ~95% confluence and then transfected with c-NKCC2 and FL-NKCC2. Stable clones were selected in culture medium supplemented with 1 mg/ml geneticin. LLC-PK1 and HEK 293 cells stably transfected with c-NKCC2 and FL-NKCC2 respectively were co-transfected with CaR WT and CaR-A843E constructs and all the experiments were done 24 h after transfection. Lipofectamine 2000 (www.lifetechnologies.com) was used for transfection according to the manufacturer's instructions.

Intracellular Ca^{2+} measurements

Cells were grown on Ø40 mm glass coverslips. HEK 293 cells were loaded with 4 μM Fura-2 AM (www.lifetechnologies.com) for 20 min at 37°C in DMEM. Ringer's Solution was used to perfuse cells during the experiment containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM Glucose, 1.2 mM $CaCl_2$, pH 7.4. Unless otherwise stated, all chemicals were from Sigma–Aldrich (www.sigmaaldrich.com). R568 was from Tocris (www.tocris.com). When dimethyl sulfoxide or ethanol was used, the final solvent concentration never exceeded 0.01% or 0.1%, respectively. In fluorescence measurements, the coverslips with dye-loaded cells were mounted in a perfusion chamber (FCS2 Closed Chamber System; BIOPTECHS) and measurements were performed using an inverted microscope (Nikon Eclipse TE2000-S microscope) equipped for single cell fluorescence measurements and imaging analysis. The sample was illuminated through a 40× oil immersion objective (NA = 1.30). The Fura-2 AM loaded sample was excited at 340 and 380 nm.

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Emitted fluorescence was passed through a dichroic mirror, filtered at 510 nm (Omega Optical) and captured by a cooled CCD camera (CoolSNAP HQ; Photometrics). Fluorescence measurements were carried out using Metafluor software (Molecular Devices, MDS Analytical Technologies). The ratio of fluorescence intensities at 340 and 380 nm was normalised with respect to basal ratio observed in the absence of stimulus (R_0).

ERK activation assay

HEK 293 cells were grown to 80–90% confluence. 24 h after transfection, cells were serum starved for 24 h prior to lysis on ice in the following buffer: 150 mM NaCl, 30 mM NaF, 5 mM EDTA, 15 mM Na_2HPO_4, 15 mM pyrophosphate and 20 mM HEPES, pH 7.2, with 1% Triton X-100 and supplemented with protease and phosphatase inhibitors. Proteins were resolved by SDS-PAGE and subsequently immunoblotted using phospho-specific ERK antibody (anti-pERK 1/2, 1:1000).

Immunofluorescence

Cells grown on coverslips were fixed in methanol for 5 min. After three washes in PBS, cells were blocked in saturation buffer (1% BSA in PBS) for 20 min at RT and incubated with the primary antibodies for 2 h at RT in saturation buffer. After three washes in PBS, cells were incubated with the appropriate Alexa Fluor-conjugated secondary antibodies for 1 h at RT. Primary antibodies used were as follows: monoclonal anti-HA antibody (1:500) to detect NKCC2, polyclonal anti-c-Myc antibody to detect CaR WT and CaR-A843E (1:400). Confocal images were obtained with a laser scanning fluorescence microscope Leica TSC-SP2.

NKCC2 activation assay

Cells were preincubated for 1 h in basic medium (135 mM NaCl, 1 mM $MgCl_2$, 1 mM Na_2SO_4, 1 mM $CaCl_2$ and 15 mM Na-HEPES, pH 7.4). Cells were then incubated for 1 h in low Cl^- medium (1 mM NaCl, 1 mM $MgCl_2$, 1 mM Na_2SO_4, 1 mM $CaCl_2$, 15 mM Na-HEPES and 134 mM Na-gluconate, pH 7.4). If required, c-NKCC2 stably transfected LLC-PK1 cells expressing CaR WT were incubated with 1 μM R568 or 5 mM Ca^{2+} 10 min prior and during c-NKCC2 activation in low Cl^- medium. In the case of $CoCl_2$, 17-ODYA and aristolochic acid (all from www.sigmaaldrich.com) treatments, c-NKCC2 stably transfected LLC-PK1 cells expressing CaR WT or CaR-A843E were incubated for 1 h with these drugs in basic medium at 2/5 mM, 10 μM and 10 μM, respectively, followed by 1 h in low Cl^- medium with the same drugs concentration. After incubations, cells were scraped into ice-cold buffer (150 mM NaCl, 30 mM NaF, 5 mM EDTA, 15 mM Na_2HPO_4, 15 mM pyrophosphate and 20 mM HEPES, pH 7.2) with 1% Triton X-100 and protease and phosphatase inhibitors. After lysis on ice for 30 min, lysates were sonicated and insolubilised material was pelleted at 16200 × *g* for 30 min. Proteins were denaturated in NuPAGE LDS sample buffer with 50 mM dithiothreitol, heated at 70°C for 10 min, and resolved on NuPAGE gels. After transfer on polyvinylidene difluoride membrane, lanes were probed with antibody against phosphorylated NKCC2 (R5, 1:500) and CaR WT/A843E (anti c-Myc, 1:500).

Statistical analysis

Data are represented as means ± S.E.M. Significant differences between means were tested by one-way analysis of variance with Newman–Keuls's post-test using GraphPad Prism software (version 5.00 GraphPad Software). $P < 0.05$ was considered to be statistically significant.

Author contribution

A.G., S.T. and F.R performed experiments and analysed the data; M.C., A.G., G.P. and M.S. designed the experiments, wrote and critically revised the paper; L.D. gave fundamental technical support for imaging experiments, G.N.H. prepared the CaR-A843E and critically revised the paper.

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Conflict of interest statement

The authors have declared no conflict of interest.

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