J Membrane Biol (2014) 247:729–737
DOI 10.1007/s00232-014-9698-0

Calcium Rapidly Down-Regulates Human Renal Epithelial Sodium Channels Via a W-7-Sensitive Mechanism

Gerard G. Robins · Geoffrey I. Sandle

Received: 19 February 2014 / Accepted: 7 June 2014 / Published online: 1 July 2014

Abstract Increases in intracellular calcium (Ca^{2+}) inhibit renal sodium (Na^+) absorption in cortical collecting ducts, but the precise mechanism is unclear. We, therefore, studied the effects of raising intracellular Ca^{2+} (using 10 μmol/L A23187, a Ca^{2+} ionophore) on wild-type and Liddle-mutated human epithelial Na^+ channels (hENaC) expressed in *Xenopus* oocytes, using the dual-electrode voltage clamp technique. A23187 decreased amiloride-sensitive Na^+ current by 55 % in oocytes expressing wild-type hENaC, an effect prevented by co-exposure to 50 μmol/L W-7 (to inhibit the Ca^{2+}/calmodulin complex). By contrast, co-exposure to 50 μmol/L calphostin (to inhibit protein kinase C) or 5 μmol/L KN-62 (to inhibit Ca^{2+}/calmodulin-dependent protein kinase II) had no effect on the decrease in amiloride-sensitive Na^+ current elicited by A23187 alone. Whereas A23187 reduced amiloride-sensitive Na^+ current in oocytes expressing wild-type hENaC, it had no similar effect in those expressing Liddle-mutated hENaCs, suggesting that the activity of individual Na^+ channels in situ was unchanged by the rise in intracellular Ca^{2+}. These data suggest that the A23187-induced rise in intracellular Ca^{2+} inhibited wild-type hENaC through a W-7-sensitive mechanism, which likely reflected enhanced removal of Na^+ channels from the cell membrane by endocytosis. We, therefore, propose that Na^+ absorption in cortical collecting duct cells is inhibited by Ca^{2+}, possibly when complexed with calmodulin.

G. G. Robins · G. I. Sandle (✉)
Leeds Institute of Biomedical and Clinical Sciences, St James's University Teaching Hospital, Level 7, Clinical Sciences Building, Beckett Street, Leeds LS9 7TF, UK
e-mail: g.i.sandle@leeds.ac.uk

Keywords Calcium · Endocytosis · Oocytes · Prostaglandins · Sodium channels

Introduction

Previous single channel studies in rat kidney tubular cells showed that raising intracellular Ca^{2+} inhibited amiloride-sensitive Na^+ channel (ENaC) activity in cell-attached but not excised inside-out apical membrane patches (Palmer and Frindt 1987), which suggested that Ca^{2+} has an indirect effect requiring one or more cytosolic factors. Furthermore, increasing intracellular Ca^{2+} mobilizes Nedd4, an E3 ubiquitin protein ligase, to the apical membrane (Plant et al. 1997; Staub et al. 2000). Nedd4 and related proteins (such as Nedd4-2) play a critical role in the endocytosis of membrane-bound ENaC, facilitating interaction between ubiquitin and ENaC, and thus "tagging" ENaC protein for subsequent endocytosis and lysosomal degradation. Human Nedd4 has three WW domains, which bind to PY motifs on human renal ENaC (hENaC) α-, β-, and γ-subunits, respectively. The WW3 domain is the most important mediator of the interaction between Nedd4 and hENaC (Lott et al. 2002). On the other hand, when a Liddle's mutation (βY620A) is incorporated into hENaC to disrupt the PY motifs, no WW domain binds to the β-subunit (Farr et al. 2000). This results in a marked decrease in ENaC removal/degradation, and subsequently an increase in Na^+ channel density in the apical membrane of distal nephron segments, leading to excessive tubular Na^+ absorption and the clinical features of Liddle's syndrome, a rare hereditary form of hypertension (Shimkets et al. 1994).

In the present study, we evaluated the effects of increasing intracellular Ca^{2+} on wild-type and Liddle-mutated hENaC (β- or γ-subunit mutations) expressed in

Xenopus oocytes. We also evaluated various agents which are known to interfere with intracellular Ca^{2+} signaling pathways by determining their effects on Na^+ channels cycling normally into and out of the membrane (wild-type hENaC). Our results suggest that raising intracellular Ca^{2+} inhibits wild-type hENaC (but not Liddle-mutated hENaCs) by stimulating endocytotic removal of Na^+ channels from the cell membrane via a W-7-sensitive mechanism, thereby implicating calmodulin. Extrapolating these findings to renal cortical collecting duct cells, we speculate that inhibition of Na^+ absorption by Ca^{2+}-dependent agents such as prostaglandin E_2 (Stokes and Kokko 1977; Hébert et al. 1991; Breyer and Breyer 2000), involves removal of apical Na^+ channels through a mechanism involving the Ca^{2+}/calmodulin complex.

Methods

Preparation of cDNA Constructs and Microinjection into *Xenopus* Oocytes

Human renal ENaC (hENaC) subunit cDNAs were incorporated into pMT3 vector (a gift of Dr. P. Snyder, University of Iowa, USA). The vector contained one of the three wild-type subunits, the β-subunit with a Liddle-type truncation (β566X), or the γ-subunit with a Liddle-type truncation (γ576X). Clones were amplified by transforming competent *Escherichia coli* grown on LB-ampicillin agar plates, the pMT3 vector being ampicillin-resistant. Plasmids were prepared using a proprietary kit (QIAGEN). Female *Xenopus laevis* (European *Xenopus* Resource Centre, University of Portsmouth, Portsmouth, UK) were killed by a schedule 1 method approved by the UK Home Office. Ovaries were removed, washed in modified Barth's saline (MBS), and divided into clumps of 10–30 oocytes, which were separated using Ca^{2+}-free Ringer's solution containing 1 mg/ml collagenase, as described previously (Canessa et al. 1993). Oocytes at Dumont stages V and VI were transferred to 96-well plates containing MBS, centrifuged (2100 rpm, 15 min), and the nuclei microinjected with either 20 nl of sterile distilled water, or 20 nl of sterile distilled water containing (3.5 ng of each subunit cDNA) wild-type hENaC, hENaC with the Liddle-mutated β-subunit, or hENaC with the Liddle-mutated γ-subunit. Injected oocytes were transferred to 24-well plates containing MBS (96 mmol/L Na^+), and incubated at 19 °C for 24–48 h.

Dual-Electrode Voltage Clamp Recording

Oocytes were superfused (1 ml/min) with a solution containing (in mmol/L): Na^+ gluconate 100, Ca^{2+} 0.38, Mg^{2+} 0.47, Cl^- 11.7, and HEPES 4.6 (pH 7.4), with Ba^{2+} 5.0 and tetraethylammonium 10 to block endogenous K^+ channels. Oocytes were impaled with the voltage and current electrodes (tip resistances <1 MΩ) fabricated from glass microcapillary tubing, and back-filled with 3 mol/L KCl. Experiments were done at room temperature (20–22 °C). When membrane voltage was stable, command voltages (−140 to +40 mV in 20 mV increments) were applied for 500 ms from a holding voltage of −10 mV, using a Labmaster TL40 interface and pClamp 5.6 software (Axon Instruments Inc., Union City, CA, USA). Whole-cell currents were measured twice, filtered at 100 Hz, averaged, and stored for later analysis. The protocol was repeated after exposing oocytes to 10 μmol/L amiloride for 30 s. This relatively high concentration of amiloride was used to ensure maximal inhibition of whole-cell Na^+ currents in oocytes expressing hENaC (Canessa et al. 1993). Differences between the pre- and post-amiloride whole-cell currents at each command voltage were taken to reflect whole-cell Na^+ currents. After washing off amiloride, an additional set of whole-cell current measurements was made at each command voltage.

Agents Used to Study hENaC Regulation by Ca^{2+}

To determine the effects of increasing intracellular Ca^{2+}, amiloride-sensitive whole-cell currents were measured using the above protocol, first in the absence and then after exposing the oocytes to 10 μmol/L A23187 (a Ca^{2+} ionophore) for 30 min, A23187 having previously been shown to raise intracellular Ca^{2+} in *Xenopus* oocytes (Boton et al. 1990). In preliminary experiments using oocytes expressing wild-type hENaC, the changes in amiloride-sensitive whole-cell currents produced by the A23187-induced increase in intracellular Ca^{2+} had reached a steady state at this time point. To determine whether increased intracellular Ca^{2+} changed hENaC activity via calmodulin, protein kinase C (PKC), or Ca^{2+}/calmodulin-dependent protein kinase II, we performed similar experiments with specific inhibitors of these regulators in the A23187-containing superperfusing solution. Thus, 50 μmol/L W-7 was used as a specific inhibitor of the Ca^{2+}/calmodulin complex (Masson et al. 1992; Blődow et al. 2003), 50 μmol/L calphostin to specifically inhibit PKC (Hartzell and Rinderknecht 1996), and 5 μmol/L KN-62 to specifically inhibit Ca^{2+}/calmodulin-dependent protein kinase II (Tohda et al. 1991; Okazaki et al. 1994). Time control experiments were performed with cDNA-injected oocytes to exclude possible "run-down" of the whole-cell Na^+ currents.

Statistical Analyses

Data are shown as mean ± SEM. Normalised amiloride-sensitive whole-cell Na^+ currents at –100 mV were compared using either Student's *t* test (for two sets of data), or one-way

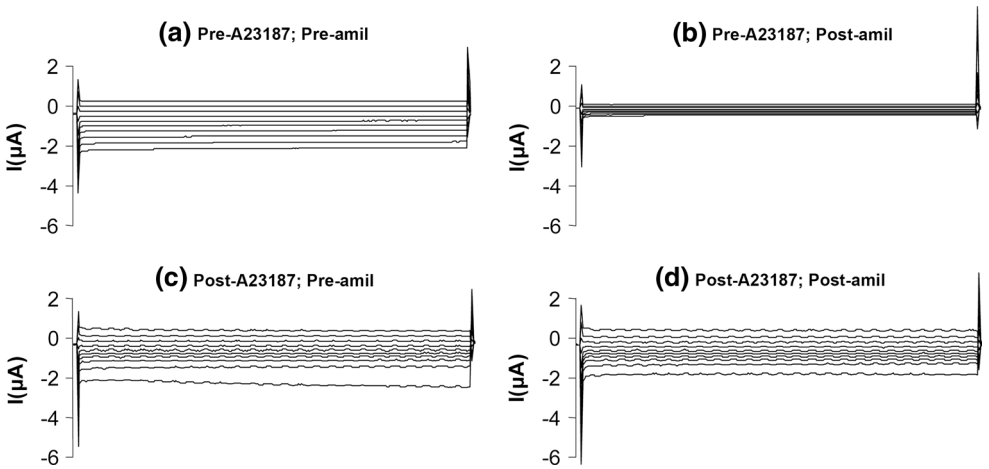

Fig. 1 Whole-cell currents (command voltages applied for 500 ms between −140 and +40 mV in 20 mV increments) in an oocyte expressing wild-type ENaC **a** in the absence of A23187 and amiloride, **b** in the absence of A23187 but in the presence of amiloride, **c** in the presence of A23187 but in the absence of amiloride, and **d** in the presence of both A23187 and amiloride. A23187 markedly decreased the oocyte's amiloride sensitivity, consistent with loss of Na^+ channel function

ANOVA with post hoc analysis (for more than two sets of data). Current–voltage relationships were compared using repeated measures ANOVA. $P < 0.05$ was taken to indicate a statistically significant difference between two mean values. Data were analyzed using SPSS for Windows (release 10.1).

Results

Effect of A23187 on Wild-Type Human Renal ENaC

Initial experiments were done in oocytes expressing wild-type hENaC to evaluate the changes in amiloride-sensitive whole-cell currents produced by 30 min exposure to 10 μmol/L A23187. A representative experiment shows that amiloride largely abolished the pre-A23187 whole-cell currents (Fig. 1a, b), consistent with Na^+ channel inhibition. By contrast, whereas the pre-A23187 and post-A23187 whole-cell currents were similar in the absence of amiloride (Fig. 1a, c), the post-A23187 whole-cell currents were relatively insensitive to amiloride (Fig. 1c, d). With each oocyte, normalisation of the post-A23187 data to data obtained in the basal (pre-A23187) state, indicated that A23187 decreased amiloride-sensitive Na^+ currents in oocytes expressing wild-type hENaC by 55 ± 10 % at −100 mV ($n = 10$, $P < 0.015$), whereas there was no change in cDNA-injected time control oocytes ($n = 12$).

Effect of A23187 + W-7 on Wild-Type Human Renal ENaC

Calmodulin is the main intracellular Ca^{2+} receptor in eukaryotic cells (Means et al. 1991), and W-7 inhibits the Ca^{2+}/calmodulin complex. The protocol with A23187 was, therefore, repeated using oocytes expressing wild-type hENaC ($n = 10$), which were bathed in a superfusing solution containing both 10 μmol/L A23187 and 50 μmol/L W-7. As shown in a representative experiment, amiloride again decreased the pre-A23187 + W-7 whole-cell currents (Fig. 2a, b), reflecting marked Na^+ channel inhibition. However, in contrast to the muted response seen in the presence of A23187 alone (Fig. 1c, d), amiloride elicited substantial decreases in whole-cell currents under post-A23187 + W-7 conditions (Fig. 2c, d).

Effect of A23187 + Calphostin C on Wild-Type Human Renal ENaC

The effects of intracellular Ca^{2+} on cell function are mediated, at least in part, by protein kinase C (PKC) (Thomas et al. 1996). In further experiments, the protocol with A23187 in oocytes expressing wild-type hENaC was repeated with both 10 μmol/L A23187 and 50 μmol/L calphostin C (a PKC inhibitor) in the superfusing solution ($n = 5$). A representative experiment shows that amiloride decreased the pre-A23187 + calphostin C whole-cell currents by inhibiting Na^+ channels (Fig. 3a, b), the pre-A23187 + calphostin C and post-A23187 + calphostin C whole-cell currents were similar in the absence of amiloride (Fig. 3a, c), and post-A23187 + calphostin C whole-cell currents were relatively insensitive to amiloride (Fig. 3c, d). A23187 + calphostin C produced a 43 ± 8 % overall decrease in the amiloride-sensitive Na^+ current.

Effect of A23187 + KN-62 on Wild-Type Human Renal ENaC

Having implicated calmodulin in the A23187-induced decrease in wild-type ENaC activity, experiments were performed ($n = 8$) to determine the role of Ca^{2+}/

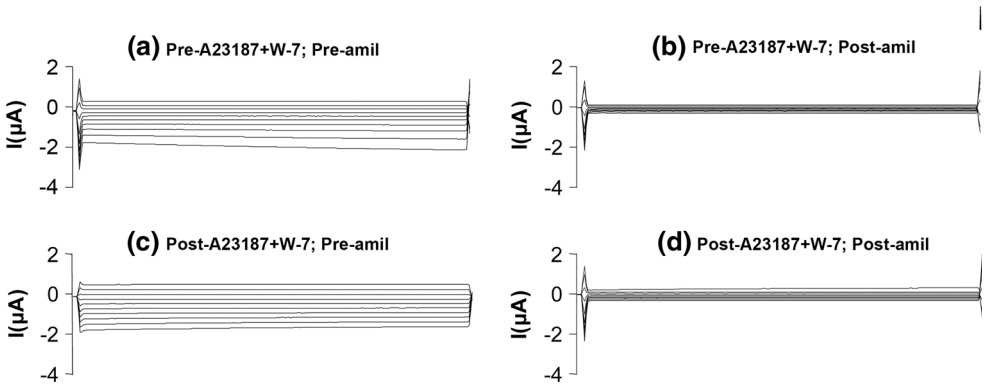

Fig. 2 Whole-cell currents (command voltages applied for 500 ms between −140 mV and +40 mV in 20 mV increments) in an oocyte expressing wild-type ENaC **a** in the absence of A23187 + W-7 and amiloride, **b** in the absence of A23187 + W-7 but in the presence of amiloride, **c** in the presence of A23187 + W-7 but in the absence of amiloride, and **d** in the presence of both A23187 + W-7 and amiloride. W-7 prevented A23187 from decreasing amiloride-sensitive Na^+ currents (see Fig. 1)

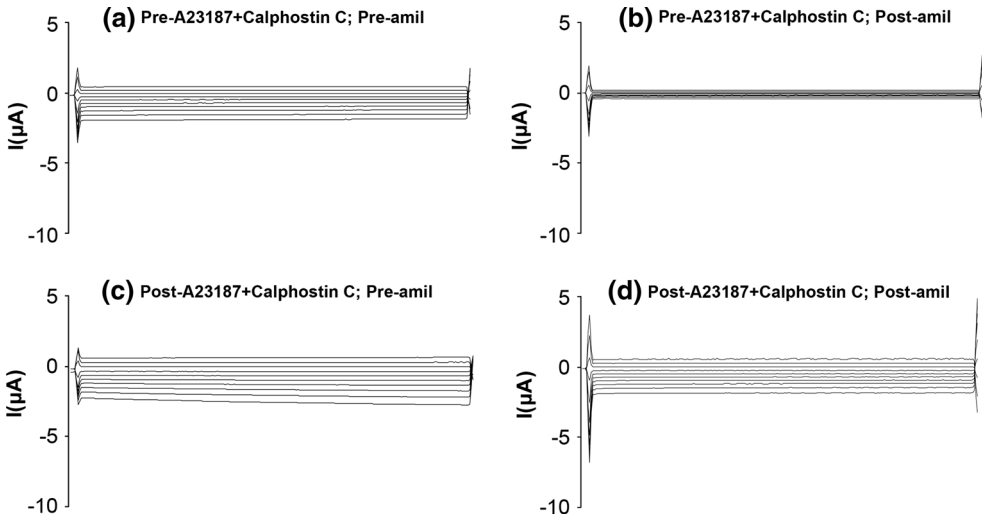

Fig. 3 Whole-cell currents (command voltages applied for 500 ms between −140 and +40 mV in 20 mV increments) in an oocyte expressing wild-type ENaC **a** in the absence of A23187 + calphostin C and amiloride, **b** in the absence of A23187 + calphostin C but in the presence of amiloride, **c** in the presence of A23187 + calphostin C but in the absence of amiloride, and **d** in the presence of both A23187 + calphostin C and amiloride. Calphostin C had no effect on the ability of A23187 to decrease amiloride-sensitive Na^+ currents (see Fig. 1)

calmodulin-dependent protein kinase II, which is inhibited by KN-62. A representative experiment in an oocyte expressing wild-type hENaC shows that, whereas amiloride largely abolished the pre-A23187 + KN-62 whole-cell currents (Fig. 4a, b), and the post-A23187 + KN-62 whole-cell currents were relatively insensitive to amiloride (Fig. 4c, d), A23187 + KN-62 produced a 57 ± 8 % overall decrease in the amiloride-sensitive Na^+ current.

Summary of Wild-Type Human Renal ENaC Data

The normalised amiloride-sensitive Na^+ currents from each protocol are summarized in Fig. 5. In each case, the left-hand bar indicates normalised data at time zero in time control experiments, and in the presence of A23187 alone or in combination with either W-7, calphostin C, or KN-62. In contrast to the time control experiments, A23187 elicited a 55 % decrease in amiloride-sensitive Na^+ current ($P < 0.03$), whereas there was no significant change with A23187 + W-7 ($P > 0.6$), and post hoc analysis indicated that the responses to A23187 alone and A23187 + W-7 were significantly different ($P < 0.035$). Addition of A23187 + calphostin C and A23187 + KN-62 produced decreases in amiloride-sensitive Na^+ current of 43 % ($P < 0.01$) and 57 % ($P < 0.001$), respectively, and one-way ANOVA followed by post hoc analyses indicated that neither calphostin C nor KN-62 altered the effect of A23187. These data show that the A23187-induced increase in intracellular Ca^{2+} produced significant

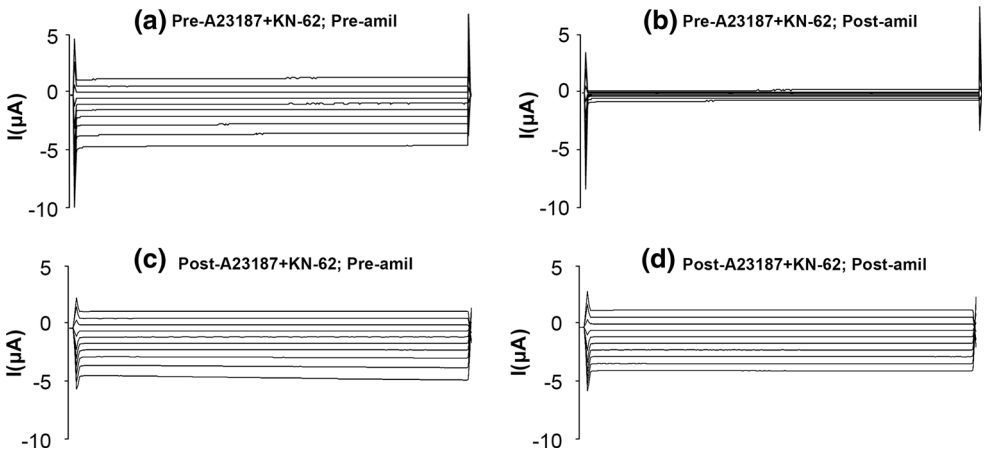

Fig. 4 Whole-cell currents (command voltages applied for 500 ms between −140 and +40 mV in 20 mV increments) in an oocyte expressing wild-type ENaC **a** in the absence of A23187 + KN-62 and amiloride, **b** in the absence of A23187 + KN-62 but in the presence of amiloride, **c** in the presence of A23187 + KN-62 but in the absence of amiloride, and **d** in the presence of both A23187 + KN-62 and amiloride. KN-62 had no effect on the ability of A23187 to decrease amiloride-sensitive Na^+ currents (see Fig. 1)

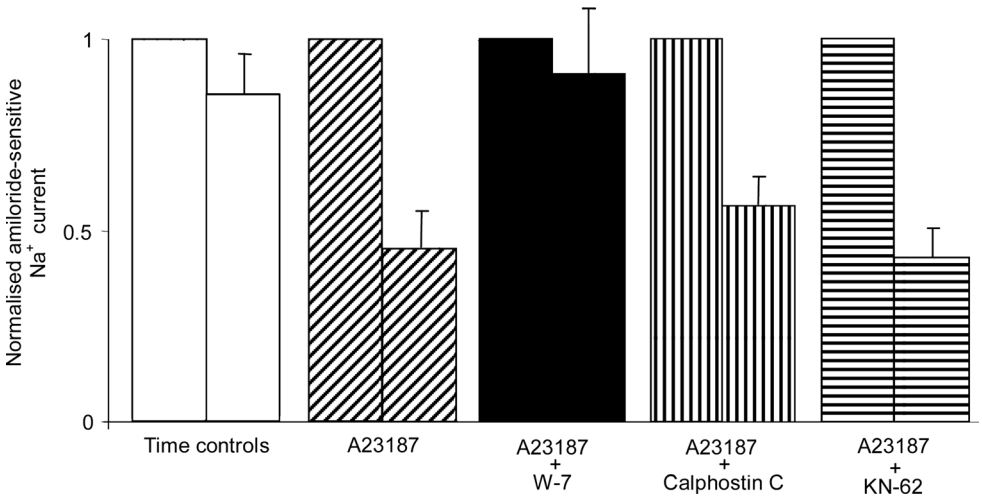

Fig. 5 Summary of amiloride-sensitive whole-cell Na^+ currents (recorded at −100 mV) in oocytes expressing wild-type ENaC in time controls (*open bars*, $n = 12$), and 30 min after the addition of 10 μmol/L A23187 (*diagonally hatched bars*, $n = 10$), 10 μmol/L A23187 + 50 μmol/L W-7 (*solid bars*, $n = 10$), 10 μmol/L A23187 + 50 μmol/L calphostin C (*vertically hatched bars*, $n = 5$), or 10 μmol/L A23187 + 5 μmol/L KN-62 (*horizontally hatched bars*, $n = 8$)

down-regulation of wild-type Na^+ channels. The ability of W-7 to prevent the A23187-induced decrease in amiloride-sensitive Na^+ current suggests that intracellular Ca^{2+} regulates these channels in conjunction with calmodulin. By contrast, calphostin C and KN-62 had no effect on the ability of A23187 to decrease amiloride-sensitive Na^+ currents, and post hoc analysis indicated that amiloride-sensitive Na^+ currents in oocytes exposed to A23187 alone and those exposed to A23187 + calphostin C were significantly lower than in time controls ($P < 0.045$ in both cases). Post hoc analysis also revealed that the decreases in amiloride-sensitive Na^+ currents in oocytes exposed to A23187 alone and those exposed to A23187 + KN-62 were comparable ($P = 0.669$). Taken together, these data suggest that neither PKC nor Ca^{2+}/calmodulin-dependent protein kinase II is likely to be involved in the down-regulation of wild-type Na^+ channels produced by A23187-induced increases in intracellular Ca^{2+}.

In addition to decreasing the amiloride-sensitive Na^+ current, A23187 increased the *amiloride-insensitive* currents in oocytes expressing wild-type hENaC (compare Fig. 1d with Fig. 1b). These may reflect Cl^- currents, since oocytes exhibit a variable endogenous Ca^{2+}-activated Cl^- current (Miledi and Parker 1984; Miledi et al. 1989), which is enhanced by intracellular Ca^{2+} injection and diminished by intracellular injection of a Ca^{2+} chelator (Miledi and Parker 1984). Our superfusing solution contained only 11.7 mmol/L Cl^-, but the intra-oocyte Cl^- concentration is likely to have been higher owing to leakage of Cl^- ions from the voltage and current micropipettes, which were filled with 3 mol/L KCl. As shown in Table 1, the normalised *amiloride-insensitive* currents at –100 mV were increased in all experiments in which A23187 was added, apart from those in which KN-62 was also present. This may reflect the inhibitory effect of KN-62 on Cl^- channel

Table 1 Normalised *amiloride-insensitive* currents in *Xenopus* oocytes expressing either wild-type hENaC (exposed to A23187 alone or in combination with W-7, calphostin C or KN-62) or Liddle-mutated hENaC (exposed to A23187 alone)

	Normalised *amiloride-insensitive* current at −100 mV (mean ± SEM)
Time controls ($n = 12$)	1.01 ± 0.10
Wild-type hENaC + A23187 ($n = 10$)	1.30 ± 0.11
Wild-type hENaC + A23187 + W-7 ($n = 10$)	1.33 ± 0.25
Wild-type hENaC + A23187 + calphostin C ($n = 5$)	1.28 ± 0.17
Wild-type hENaC + A23187 + KN-62 ($n = 8$)	0.95 ± 0.12
β-Liddle hENaC + A23187 ($n = 7$)	2.30 ± 0.51
γ-Liddle hENaC + A23187 ($n = 6$)	1.37 ± 0.20

activation by Ca^{2+}/calmodulin-dependent protein kinase II, as previously reported in *Xenopus* oocytes (Tohda et al. 1991).

Effect of A23187 on Liddle-Mutated Human Renal ENaC

Basal amiloride-sensitive Na^+ currents in oocytes expressing β-subunit Liddle-mutated hENaC ($n = 7$) were similar to those in oocytes expressing γ-subunit Liddle-mutated hENaC ($n = 6$), and in both cases (Fig. 6), these currents were substantially greater than those in oocytes expressing wild-type hENaC (Fig. 1a). In contrast to its inhibitory effect on wild-type hENaC, A23187 had no effects on amiloride-sensitive Na^+ currents in oocytes expressing either β-subunit Liddle-mutated hENaC or γ-subunit Liddle-mutated hENaC (Fig. 6), which suggests that the $COOH^-$ termini of the wild-type hENaC β- and γ-subunits possess critical Ca^{2+} regulatory sites. It should be noted that the increases in *amiloride-insensitive* currents in oocytes expressing the Liddle-mutated hENaCs (see Table 1) are not apparent in Fig. 6, because the current scales are much larger than those for the wild-type hENaC experiments.

Discussion

Previous patch clamp studies demonstrated that raising the concentration of Ca^{2+} inhibited Na^+ channel activity in cell-attached patches but not in excised inside-out patches, which suggested that Ca^{2+} acted in conjunction with one or more additional intracellular factors (Palmer and Frindt 1987). The results of the present study performed in *Xenopus* oocytes are consistent with the view that the acute inhibitory effect of Ca^{2+} on wild-type Na^+ channels involves calmodulin, whereas neither PKC nor Ca^{2+}/calmodulin-dependent protein kinase II appears to have a significant role. This Ca^{2+}-induced decrease in wild-type Na^+ channel activity reflects cellular events which clearly differ from those underlying the rapid (<5 min) and sustained PKC-dependent Ca^{2+} influxes elicited by aldosterone in renal and colonic cells lines, which activate basolateral Na^+/H^+ exchange and basolateral K^+ channels as a prelude to the later (after ∼4 h) enhanced expression of Na^+ channels at the cell surface (Doolan and Harvey 1996; Harvey and Higgins 2000). Although we did not measure the increases in intra-oocyte Ca^{2+} produced by A23187, this ionophore is known to raise intracellular Ca^{2+} in *Xenopus* oocytes (Boton et al. 1990).

The ability of the A23187-induced increase in intracellular Ca^{2+} to decrease amiloride-sensitive Na^+ currents in oocytes expressing wild-type hENaC could, in theory, have reflected decreases in Na^+ channel open probability and/or single channel conductance. Although we did not test this by single channel recording, two sets of experimental findings make these possibilities seem unlikely. First, increasing intracellular Ca^{2+} by use of ionomycin inhibited the activity of rat ENaC expressed in Madin–Darby canine kidney cells, an effect which did not involve a decrease in single Na^+ channel conductance (Ishikawa et al. 1998). Second, our novel observation that A23187 had no effect on amiloride-sensitive Na^+ currents in oocytes expressing β-subunit or γ-subunit Liddle-mutated hENaC, strongly suggests that raising intra-oocyte Ca^{2+} did not change the unitary properties of Liddle-mutated Na^+ channels, nor would we have expected such changes in wild-type Na^+ channels. Our results suggest that Ca^{2+}, acting with calmodulin, decreased amiloride-sensitive Na^+ currents in oocytes expressing wild-type hENaC by promoting Na^+ channel removal from the cell membrane via endocytosis. Endocytosis requires Nedd4, a ubiquitin protein ligase which binds to proline-rich regions of the wild-type Na^+ channel β- and γ-subunits as a prerequisite for channel removal from the membrane (Staub et al. 1996), and when co-expressed with wild-type ENaC in *Xenopus* oocytes, results in down-regulation of the channels (Abreil et al. 1999; Goulet et al. 1998). By contrast, Nedd4 has no effect when co-expressed with a Liddle-mutated ENaC because they lack PY motifs and have an inherent resistance to ubiquitination (Abreil et al. 1999). Nedd4 possesses a C2 domain, those present in other proteins binding to cell membrane phospholipids in a Ca^{2+}-dependent manner

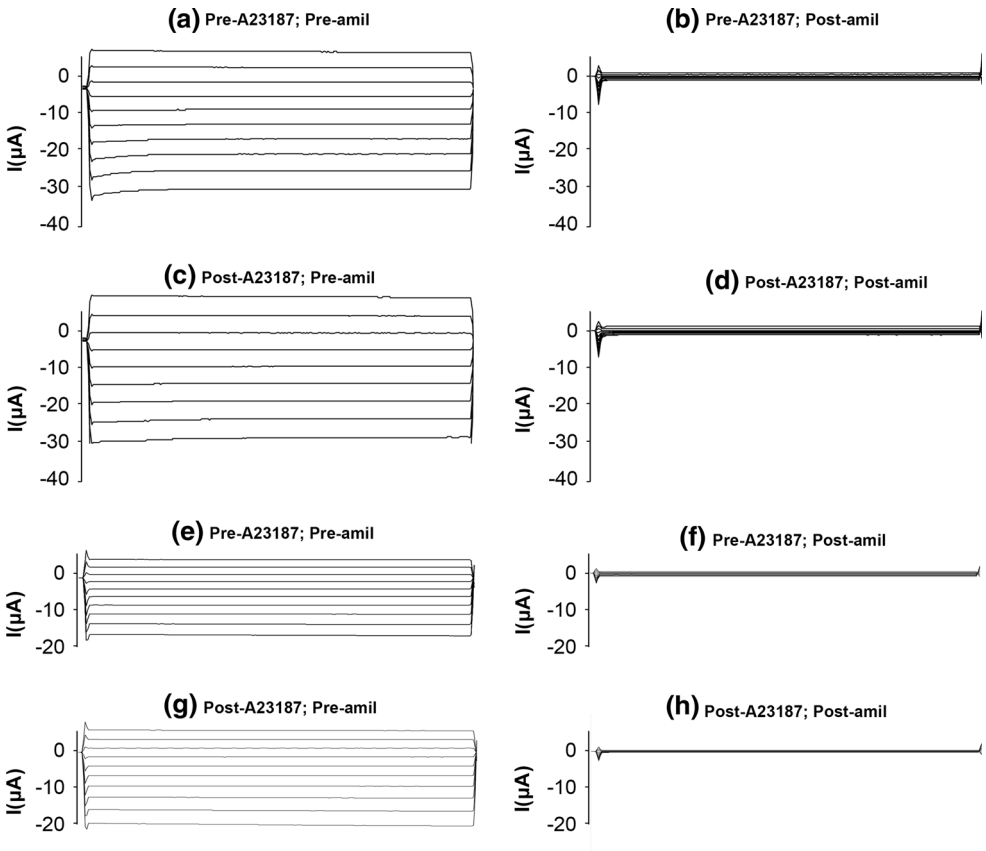

Fig. 6 Whole-cell currents (command voltages applied for 500 ms between −140 and in 20 mV increments) in oocytes expressing β-subunit Liddle-mutated hENaC (**a**–**d**) or γ-subunit Liddle-mutated hENaC (**e**–**h**), **a**, **e** in the absence of A23187 and amiloride, **b**, **f** in the absence of A23187 but in the presence of amiloride, **c**, **g** in the presence of A23187 but in the absence of amiloride, and **d**, **h** in the presence of both A23187 and amiloride. Amiloride-sensitive Na^+ currents in oocytes expressing the Liddle mutations were substantially greater than in those expressing wild-type ENaC, and were not changed by A23187

(Nalefski and Falke 1996). Ca^{2+}-dependent translocation of Nedd4 (mainly to the apical membrane) occurs within 5 min in MDCK cells, and deletion of the C2 domain prevents membrane-targeted localization of Nedd4 (Plant et al. 1997). Thus, it seems likely that if increasing intra-oocyte Ca^{2+} by A23187 triggered rapid mobilization of endogenous Nedd4 (or a homologue such as Nedd4-2) to the membrane of oocytes expressing wild-type hENaC (as seen in MDCK cells (Plant et al. 1997)), then the resulting decrease in amiloride-sensitive Na^+ current will have reflected endocytosis. On the other hand, in oocytes expressing either β-subunit or γ-subunit Liddle-mutated hENaC, both of which lacked the Nedd4 binding PY motifs, Nedd4 will have failed to initiate endocytosis, irrespective of the rise in intra-oocyte Ca^{2+}.

We set out to test the effect of increasing intracellular Ca^{2+} (elicited by A23187) on hENaC in *Xenopus* oocytes. Our results indicate that the down-regulatory effect of intracellular Ca^{2+} on wild-type (but not Liddle-mutated) hENaC is W-7-sensitive (and by inference, calmodulin-dependent), without the involvement of PKC or Ca^{2+}/calmodulin protein kinase II. Other studies in epithelial cells expressing endogenous ENaC, which were not designed specifically to evaluate the effect of raising intracellular Ca^{2+} have provided results which in some respects are at variance with our own. For example, activation of PKC by phorbol-12-myristate 13-acetate (PMA) decreased Na^+ channel activity by 67 % in rat cortical collecting tubule cells, the effect being abolished by staurosporine, an inhibitor of PKC and Ca^{2+}/calmodulin-dependent protein kinase II (Frindt et al. 1996). Furthermore, staurosporine prevented the ability of ouabain to markedly down-regulate Na^+ activity in these cells, whereas KN-62 (a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II) had no effect on the ouabain-induced decrease in Na^+ activity (Frindt et al. 1996). Ca^{2+}-dependent activation of apical PKC also inhibited apical Na^+ channels in rabbit cortical collecting tubules (Ling et al. 1992). In cultured renal A6 cells, in which Na^+ channel activity and benzamil-sensitive Na^+ transport were stimulated by hypotonicity (which itself increases intracellular Ca^{2+}; Rothstein and Mack 1992), W-7 significantly decreased benzamil-sensitive Na^+ transport irrespective of whether it was applied before or after hypotonic stimulation, whereas application of KN-93 (an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II) before hypotonic stimulation decreased benzamil-sensitive Na^+ transport by ~60 %, but had no significant effect when applied after the hypotonic stimulus (Tokuda et al. 2002). Thus, under those specific experimental conditions, Ca^{2+}/calmodulin-dependent protein kinase II appeared to be

involved in Na^+ channel translocation, whereas the maintenance of hypotonicity-induced Na^+ channel activity was calmodulin-dependent (Tokuda et al. 2002). Although these other studies tested Ca^{2+}-dependent signaling pathways, they were not designed to evaluate the effect of raising intracellular Ca^{2+} per se, whereas our results are consistent with A23187 having produced a marked and sustained increase in intra-oocyte Ca^{2+} which down-regulated wild-type hENaC through a mechanism which was inhibited by W-7, and therefore, predominantly (if not solely) calmodulin-dependent. However, additional factors should be taken into consideration when interpreting our data. Thus, at the concentration used in the present study to inhibit Ca^{2+}/calmodulin (50 μmol/L), the amphipathic weak base W-7 almost certainly attached to the inner leaflet of the plasma membrane and decreased its net negative charge, which may have altered the function of myristolated alanine-rich C kinase substrate (MARCKS) (Sengupta et al. 2007). MARCKS possesses sites for PKC phosphorylation and Ca^{2+}/calmodulin binding (Hartwig et al. 1992), and also acts as an adaptor protein which binds to and presents phosphatidylinositol phosphates (PIPs; particularly phosphatidylinositol 4,5-biphosphate, PIP_2) to regulate ENaC activity (Alli et al. 2012). In *Xenopus* 2F3 renal epithelial cells, MARCKS colocalised with PIP_2 at the apical membrane, but translocated to the cytoplasm after PKC stimulation or raising intracellular Ca^{2+} with ionomycin, whilst PKC-induced MARCKS phosphorylation decreased amiloride-sensitive transepithelial ENaC currents, and inhibition of PKC stimulated ENaC activity by allowing unphosphorylated MARCKS to bind PIPs for presentation to ENaC (Alli et al. 2012). Putting our results into context with those from *Xenopus* 2F3 cells, A23187 alone should have activated PKC, as well as raising intracellular Ca^{2+} per se, both these effects promoting translocation into the cytoplasm of any MARCKS present in the oocyte membrane and decreasing PIP-regulated wild-type hENaC activity. W-7 might also have been expected to decrease wild-type hENaC activity by impairing the functionality of MARCKS (Sengupta et al. 2007). However, W-7 prevented the A23187-induced decrease in amiloride-sensitive Na^+ current, whilst the PKC inhibitor calphostin C had no effect, which is consistent with our view that the decrease in amiloride-sensitive Na^+ current induced by A23187 reflected predominantly Ca^{2+}/calmodulin-dependent endocytosis of wild-type hENaC. Furthermore, the A23187-induced rise in intracellular Ca^2 had no effect on amiloride-sensitive Na^+ currents in oocytes expressing the two Liddle-mutated hENaCs, which suggests that MARCKS had no significant role in regulating basal mutant hENaC activity via PIPs.

It is of interest that A23187 also increased the *amiloride-insensitive* currents in oocytes expressing wild-type hENaC (compare Fig. 1d with Fig. 1b). Increased *amiloride-insensitive* currents have previously been exploited as an intrinsic marker of Ca^{2+} entry into *Xenopus* oocytes (Boton et al. 1990; Bourinet et al. 1992), since oocytes demonstrate variable endogenous Ca^{2+}-activated Cl^- currents (Miledi and Parker 1984; Miledi et al. 1989). These *amiloride-insensitive* currents may well reflect the large leak current previously reported in *Xenopus* oocytes on removal of external Ca^{2+}, which was mediated by ~90 pS Cl^- channels (Weber et al. 1995). We found that the normalised *amiloride-insensitive* currents were increased whenever A23187 was added, apart from those experiments in which KN-62 was also present, which may have inhibited Cl^- channel activation by Ca^{2+}/calmodulin-dependent protein kinase II, as previously reported in *Xenopus* oocytes (Tohda et al. 1991).

Our finding that raising intracellular Ca^{2+} rapidly inhibits Na^+ channels via what appears to be a calmodulin-dependent mechanism in *Xenopus* oocytes expressing wild-type hENaC, but has no effect in those expressing Liddle-mutated hENaCs, suggests that the proposed Ca^{2+}/calmodulin complex acts at sites on the $COOH^-$ termini of wild-type β- and γ-subunits to increase the rate of Na^+ channel drop-out by endocytosis. In terms of the regulation of renal Na^+ excretion, we speculate that this mechanism may underlie the inhibitory effect of endogenous prostaglandin E_2 (PGE_2) on renal Na^+ channels (Breyer and Breyer 2000). PGE_2 acts through a group of G protein-coupled (E-prostanoid or EP) receptors, four subtypes (EP_{1-4}) having been characterised (Breyer at el Breyer et al. 1996; Toh et al. 1995). The EP_1 receptor is particularly relevant to the inhibitory effect of PGE_2, since its activation increases intracellular Ca^{2+} and phosphatidylinositol bisphosphate hydrolysis (Båtshake et al. 1995; Funk et al. 1993; Watabe et al. 1993). Based on these findings, we suggest that a W-7-sensitive, Ca^{2+}-dependent mechanism may play a critical role in mediating the inhibitory effect of PGE_2 on renal Na^+ absorption.

Acknowledgments Supported by a project grant from the British Heart Foundation.

References

Abreil H, Loffing J, Rebhun JF et al (1999) Defective regulation of the epithelial Na^+ channel by Nedd4 in Liddle's syndrome. J Clin Invest 103:667–673

Alli AA, Bao HF, Alli AA et al (2012) Phosphatidylinositol phosphate-dependent regulation of *Xenopus* ENaC by MARCKS protein. Am J Physiol Renal Physiol 303:F800–F811

Båtshake B, Nilsson C, Sundelin J (1995) Molecular characterization of the mouse prostanoid EP_1 receptor gene. Eur J Biochem 231:809–814

Blődow A, Ngezahayo A, Ernst A et al (2003) Calmodulin antagonists suppress gap junction coupling in isolated Hensen cells of the guinea pig cochlea. Pflüg Arch 446:36–41

Boton R, Singer D, Dascal N (1990) Inactivation of calcium-activated chloride conductance in *Xenopus* oocytes: roles of calcium and protein kinase C. Pflüg Arch 416:1–6

Bourinet E, Fournier F, Nargeot J et al (1992) Endogenous *Xenopus*-oocyte Ca-channels are regulated by protein kinases A and C. FEBS Lett 299:5–9

Breyer MD, Breyer RM (2000) Prostaglandin E receptors and the kidney. Am J Physiol Renal Physiol 279:F12–F23

Breyer M, Jacobson H, Breyer R (1996) Functional and molecular aspects of renal prostaglandin receptors. J Am Soc Nephrol 7:8–17

Canessa CM, Horisberger JD, Rossier BC (1993) Epithelial sodium channel related to proteins involved in neurodegeneration. Nature 361:467–470

Doolan CM, Harvey BJ (1996) Rapid effects of steroid hormones on free intracellular calcium in T84 colonic epithelial cells. Am J Physiol Cell Physiol 271:C1935–C1941

Farr TJ, Coddington-Lawson SJ, Snyder PM et al (2000) Human Nedd4 interacts with the human epithelial Na^+ channel: WW3 but not WW1 binds to Na^+ channel subunits. Biochem J 345:503–509

Frindt G, Palmer LG, Windhager EE (1996) Feedback regulation of Na channels in rat CCT. IV. Mediation by activation of protein kinase C. Am J Physiol Renal Fluid Electrolyte Physiol 270:F371–F376

Funk C, Furchi L, FitzGerald G et al (1993) Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. J Biol Chem 268:26767–26772

Goulet CC, Volk KA, Adams CM et al (1998) Inhibition of the epithelial Na^+ channel by interaction of Nedd4 with a PY motif deleted in Liddle's syndrome. J Biol Chem 273:30012–30017

Hartwig JH, Thelen M, Rosen A et al (1992) MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium/calmodulin. Nature 356:618–622

Hartzell HC, Rinderknecht A (1996) Calphostin C, a widely used protein kinase C inhibitor, directly and potently blocks L-type Ca channels. Am J Physiol Cell Physiol 270:C1293–C1299

Harvey BJ, Higgins M (2000) Nongenomic effects of aldosterone on Ca^{2+} in M-1 cortical collecting duct cells. Kidney Int 57: 1395–1403

Hébert RL, Jacobson HR, Breyer MD (1991) Prostaglandin E_2 inhibits sodium transport in rabbit cortical collecting duct by increasing intracellular calcium. J Clin Invest 87:1992–1998

Ishikawa T, Marunaka Y, Rotin D (1998) Electrophysiological characterization of the rat epithelial Na^+ channel (rENaC) expressed in MDCK cells. Effects of Na^+ and Ca^{2+}. J Gen Physiol 111:825–846

Ling BN, Kokko KE, Eaton DC (1992) Inhibition of apical Na^+ channels in rabbit cortical collecting tubules by basolateral prostaglandin E_2 is modulated by protein kinase C. J Clin Invest 90:1328–1334

Lott JS, Coddington-Lawson SJ, Teedale-Spittle PH et al (2002) A single WW domain is the predominant mediator of the interaction between the human ubiquitin-protein ligase Nedd4 and the human epithelial sodium channel. Biochem J 361:481–488

Masson M, Spezzatti B, Chapman J et al (1992) Calmodulin antagonists chlorpromazine and W-7 inhibit exogenous cholesterol esterification and sphingomyelinase activity in human skin fibroblast cultures. Similarities between drug-induced Niemann-Pick type C lipidoses. J Neurosci Res 31:844–888

Means AR, Van Berkum MF, Bagchi et al (1991) Regulatory functions of calmodulin. Pharmacol Ther 50:255–270

Miledi R, Parker I (1984) Chloride current induced by injection of calcium into *Xenopus* oocytes. J Physiol 357:173–183

Miledi R, Parker I, Woodward RM (1989) Membrane currents elicited by divalent cations in *Xenopus* oocytes. J Physiol 417:173–195

Nalefski EA, Falke JJ (1996) The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci 5:2375–2390

Okazaki K, Ishikawa T, Inui M et al (1994) KN-62, a specific Ca^{++}/calmodulin-dependent protein kinase inhibitor, reversibly depresses the rate of beating of cultured fetal mouse and cardiac myocytes. J Pharmacol Exp Ther 270:1319–1324

Palmer LG, Frindt G (1987) Effects of cell Ca and pH on Na channels from the rat cortical collecting duct. Am J Physiol Renal Physiol 253:F333–F339

Plant PJ, Yeger H, Staub O et al (1997) The C2 domain of the ubiquitin protein ligase Nedd4 mediates Ca^{2+}-dependent plasma membrane localization. J Biol Chem 272:32329–32336

Rothstein A, Mack E (1992) Volume-activated calcium uptake: its role in cell volume regulation of Madin–Darby canine kidney cells. Am J Physiol Cell Physiol 262:C339–C347

Sengupta P, Ruano MJ, Tebar F et al (2007) Membrane-permeable calmodulin inhibitors (e.g. W-7/W-13) bind to membranes, changing the electrostatic surface potential. Dual effect of W-13 on epidermal growth factor receptor activation. J Biol Chem 282:8474–8486

Shimkets RA, Warnock DG, Bositis CM et al (1994) Liddle's syndrome: heritable human hypertension caused by mutations in the β-subunit of the epithelial sodium channel. Cell 79:407–414

Staub O, Dho S, Henry PC et al (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome. EMBO J 15:2371–2380

Staub O, Abriel H, Plant P et al (2000) Regulation of the epithelial Na^+ channel by Nedd4 and ubiquitination. Kidney Int 57: 809–815

Stokes JB, Kokko JP (1977) Inhibition of sodium transport by prostaglandin E_2 across the isolated, perfused rabbit collecting tubule. J Clin Invest 59:1099–1104

Thomas AP, Bird GS, Hajnóczky G et al (1996) Spatial and temporal aspects of cellular calcium signalling. FASEB J 10:1505–1517

Toh H, Ichikawa A, Narumiya S (1995) Molecular evolution of receptors for eicosanoids. FEBS Lett 361:17–21

Tohda M, Nakamua J, Hidaka H et al (1991) Inhibitory effects of KN-62, a specific inhibitor of Ca/calmodulin-dependent protein kinase II, on serotonin-evoked Cl^- current and $^{36}Cl^-$ efflux in *Xenopus* oocytes. Neurosci Lett 129:47–50

Tokuda S, Niisato N, Morisaki S et al (2002) Calmodulin-dependent regulation of hypotonicity-induced translocation of ENaC in renal epithelial A6 cells. Biochem Biophys Res Comm 298: 619–623

Watabe A, Sugimoto Y, Irie A et al (1993) Cloning and expression of cDNA for a mouse EP_1 subtype of prostaglandin E receptor. J Biol Chem 268:20175–20178

Weber WM, Liebold KM, Reifarth FW et al (1995) The Ca^{2+}-induced leak current in *Xenopus* oocytes is indeed mediated through a Cl^- channel. J Membr Biol 148:263–275