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Rapid report

Modulation of KCNQ4 channel activity by changes in cell volume

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Abstract

KCNQ4 channels expressed in HEK 293 cells are sensitive to cell volume changes, being activated by swelling and inhibited by shrinkage, respectively. The KCNQ4 channels contribute significantly to the regulatory volume decrease (RVD) process following cell swelling. Under isoosmotic conditions, the KCNQ4 channel activity is modulated by protein kinases A and C, G protein activation, and a reduction in the intracellular Ca^{2+} concentration, but these signalling pathways are not responsible for the increased channel activity during cell swelling.

Keywords: KCNQ2/3; Cell swelling; PKA; PKC; Tyrosine phosphatase

Most cell types respond to an increase in cell volume by undergoing a regulatory volume decrease (RVD) response, which often is mediated by loss of KCl through separate K^+ and Cl^- channels [1]. Different K^+ channels, depending on cell type, have been described to play a role in the swelling-induced K^+ loss. These channels include Kv1.3 and Kv1.5 in lymphocytes, see Ref. [2], Ca^{2+}-activated K^+ channels of small conductance (SK) in, e.g. human liver cells [3], of intermediate conductance (IK) in human T lymphocytes [4] and intestine 407 cells [5] as well as big conductance (BK) channels in, e.g. rabbit kidney proximal tubule cells [6] and human osteoblasts [7]. Activation of SK and IK channels by cell swelling is also described for cloned channels expressed in *Xenopus* oocytes [8] and HEK 293 cells [9]. A TASK channel was suggested as the molecular identity of the volume-sensitive K^+ conductance in Ehrlich cells [10] and when expressed in HEK 293 cells TASK-2 was found to be sensitive to cell volume changes [11]. Finally, KCNQ1 channels are activated by cell swelling when expressed in COS 7 cells [12] and *Xenopus* oocytes [13]. Swelling-induced activation of KCNQ4 channels was suggested by a study on channels heterologously expressed in *Xenopus* oocytes [13]. In the present study, the sensitivity of KCNQ4 and KCNQ2/3 channels towards changes in cell volume was further investigated following stable expression in HEK 293 cells. Physiological regulation of KCNQ4 channels is currently poorly described, and we have thus investigated the possible regulatory role of PKA, PKC, tyrosine phosphorylation, Ca^{2+} and G proteins under iso- as well as hypo-osmotic conditions.

To examine whether KCNQ4 channels are sensitive to changes in cell volume HEK 293 cells stably expressing the channel [14] were exposed to changes in extracellular osmolarity. Since an increase in cell volume will result in the activation of a volume-sensitive Cl^- conductance ($I_{Cl,vol}$) [15], the whole-cell K^+ current (I_K) was followed over time by pulsing to the equilibrium potential for Cl^- (E_{Cl}). As seen from Fig. 1A hypoosmotic cell swelling induced a rapid increase in I_K in KCNQ4 expressing HEK 293 cells, whereas a hyperosmotic challenge completely abolished the current. In some experiments, like the one illustrated in Fig. 1A, inhibition of the KCNQ4 current by the hyperosmotic challenge appeared biphasic. However, this was not the case in other experiments, see e.g. Fig. 2A. The whole-cell Cl^- current (I_{Cl}) was followed by pulsing to E_K. Fig. 1A demonstrates that under the experimental conditions employed in the present study cell swelling activates only a small $I_{Cl,vol}$ which likewise was abolished by the hyperosmotic solution. The voltage-dependency of the whole-cell current under iso-, hypo- and hyperosmotic conditions is illustrated in Fig. 1B. The I/V curves shown in Fig. 1C were constructed at the time points indicated by arrows in Fig. 1B.

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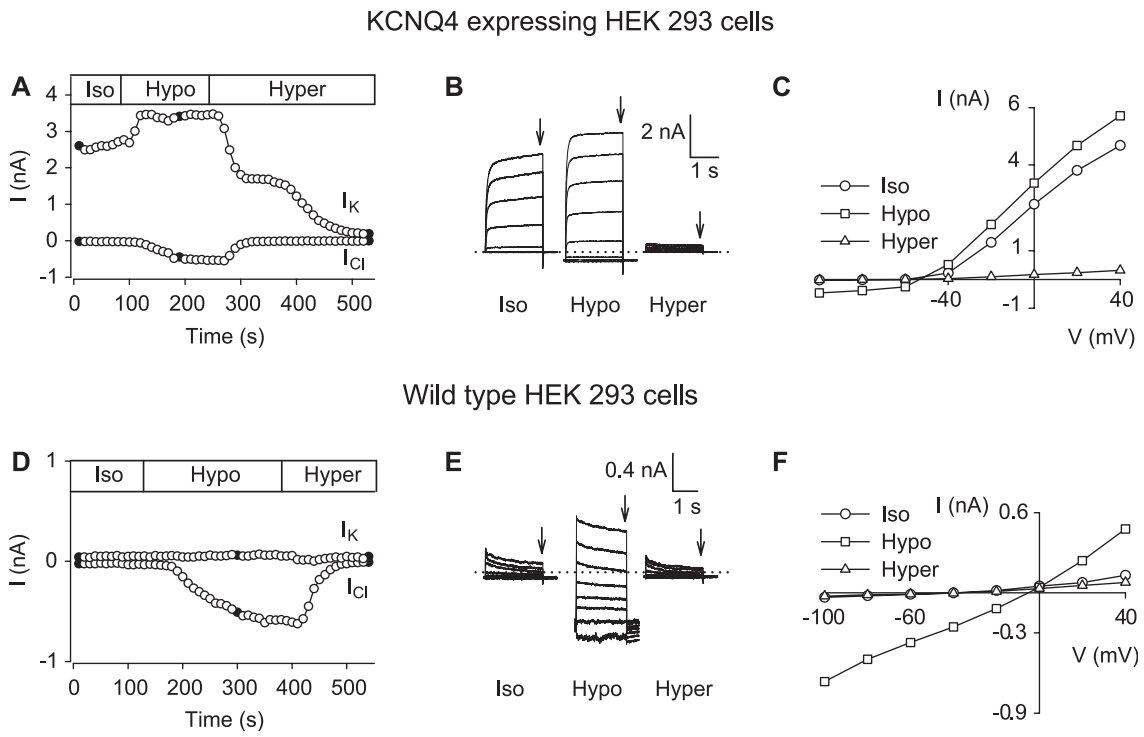

Fig. 1. Effect of osmotic changes on whole-cell K^+ (I_K) and Cl^- (I_{Cl}) currents in KCNQ4-expressing and wild type HEK 293 cells. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and 100 U/ml penicillin–100 μg/ml streptomycin at 37 °C and 5% CO_2. Standard whole-cell patch-clamp experiments were performed at 37 °C essentially as described previously [10,35]. The pipette solution contained (in mM): 2 NaCl, 8 KCl, 108 K gluconate, 1.2 $MgCl_2$, 10 HEPES, 0.1 EGTA, 1.5 Na_2ATP, pH 7.4 and adjusted to 295 mOsm using D-mannitol. Upon cell swelling, the intracellular K^+ and Cl^- concentration is calculated to be diluted to 96 and 10 mM, respectively [36]. The extracellular hypoosmotic (200 mOsm) solution was (in mM): 1 NaCl, 5 KCl, 90 Na gluconate, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.4. Isoosmotic (300 mOsm) and hyperosmotic (400 mOsm) solutions were made by addition of D-mannitol. The holding potential was −80 mV, and the following voltage protocol was applied every 10 s: a 1 s step to −78 mV (E_K), a 1 s step to −80 mV followed by a 2 s step to 0 mV (E_{Cl}). I_{Cl} was measured at a small window at E_K and I_K at the end of the 2 s pulse to E_{Cl}. (A) Time course of I_K and I_{Cl} in KCNQ4-transfected HEK 293 cells following changes in extracellular osmolarity. (B) Voltage-dependent properties of the whole-cell current in KCNQ4-transfected cells. From the holding potential of −80 mV, 20 mV steps of 2 s duration was applied from −100 to 40 mV under iso-, hypo- and hyperosmotic conditions at the time points indicated with filled symbols in Panel A. (C) Current/voltage (I/V) relationship calculated from the current traces shown in B at the time points indicated by the arrows. (D) Time-course of the whole-cell currents in wild type (non-transfected) HEK 293 cells. All experimental conditions were as in A, but please note the change in *Y*-axis. (E) Voltage-dependent properties of the whole-cell current in non-transfected cells. All experimental conditions were as in B. (F) I/V relationship calculated from the current traces shown in E at the time points indicated by the arrows. The figure is representative of 16 KCNQ4-expressing cells and four untransfected cell experiments, respectively. Mean KCNQ4 currents under iso- and hypoosmotic conditions are depicted in Fig. 4.

Under isoosmotic conditions where virtually no Cl^- current was present, stepping the potential from −100 to +40 mV resulted in a slowly activating current characteristic of KCNQ4 [14]. Hypoosmotic cell swelling activated KCNQ4 channels, inducing an increase in current at all potentials, whereas hyperosmotic cell shrinkage strongly inhibited the KCNQ4 current (Fig. 1B). The reversal potential of the current under hypoosmotic conditions is slightly shifted towards more positive potentials, indicating contribution from $I_{Cl,vol}$ as well as KCNQ4 channels. Fig. 1D–F show results from similar experiments performed on untransfected HEK 293 cells. These experiments confirm that KCNQ4 channels mediate the swelling-induced increase in I_K shown in Fig. 1A–C, as no increase in I_K is observed under hypoosmotic conditions in wild-type HEK 293 cells. The $I_{Cl,vol}$ observed is similar to that reported previously [15].

The isoosmotic as well as the swelling-induced I_K in KCNQ4 expressing HEK 293 cells was completely inhibited by the KCNQ channel inhibitors linopirdine (50 μM, $n=4$, not illustrated) and bepridil (20 μM, $n=3$, not illustrated), corroborating that the swelling-induced I_K is KCNQ4 mediated. It should be noted that bepridil besides inhibiting KCNQ4 also blocked $I_{Cl,vol}$ in these cells (not shown). The effect of bepridil on $I_{Cl,vol}$ has not been further investigated. The sensitivity of KCNQ4 channels to cell volume changes observed in the present study is in agreement with the data previously published for channels expressed in *Xenopus* oocytes [13].

Retigabine, an anticonvulsant, activates KCNQ-type channels including KCNQ4 [16,17] and KCNQ2/3 [18]. To investigate whether hypoosmotic exposure induced maximal activation of the KCNQ4 channels, retigabine was added both under iso- and hypoosmotic conditions (Fig. 2A). As expected, retigabine induced a reversible increase in I_K under isoosmotic conditions. A hypoosmotic challenge induced an increase in the KCNQ4 current amplitude, which

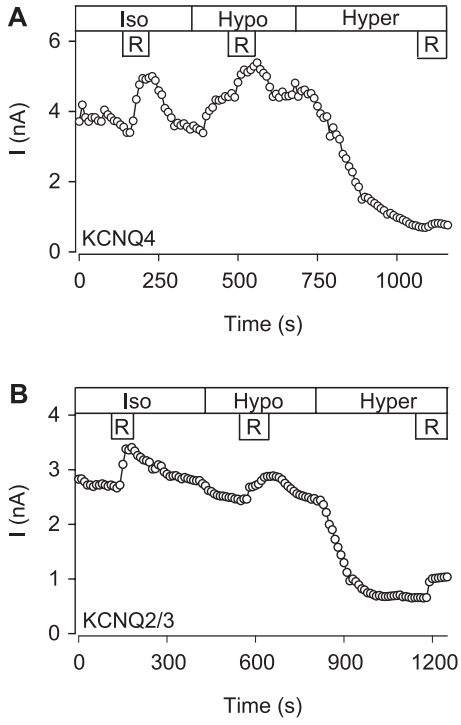

Fig. 2. Effect of the KCNQ channel opener retigabine on the KCNQ4 (A) and KCNQ2/3 (B) currents under different osmotic conditions. The whole-cell I_K was measured as described in the legend to Fig. 1. Cells were exposed to isoosmotic (300 mOsm), hypoosmotic (200 mOsm), or hyperosmotic (400 mOsm) extracellular solutions, and retigabine (10 μM) as indicated. (A, $n=3$; B, $n=4$).

was further stimulated by application of retigabine, suggesting that the degree of cell swelling induced by the present experimental conditions did not cause maximal activation of the KCNQ4 channels. During hyperosmotic conditions, where strong inhibition of KCNQ4 channels was observed, application of retigabine induced only a small increase in the KCNQ4 current level (Fig. 2A). Whether this reflects that the channels are strongly inhibited by the hyperosmotic challenge or perhaps even removed from the membrane following prolonged cell shrinkage is unclear. It should be noted that while short-term exposure (<2 min) to a hyperosmotic challenge induces a reversible inhibition of the channels, prolonged application of the hyperosmotic solution leads to an irreversible inhibition.

To examine whether the closely related KCNQ2/3 heteromeric channel complex is sensitive to cell volume changes, HEK 293 cells expressing these channels [17] were exposed to changes in extracellular osmolarity as illustrated in Fig. 2B. Unlike the KCNQ4 current, KCNQ2/3 currents were not potentiated by a hypoosmotic challenge, but these channels were still inhibited by hyperosmotic exposure. Prolonged exposure to hypoosmotic solutions (5 min) likewise failed to activate the KCNQ2/3 channels ($n=3$, not illustrated). Application of retigabine increased I_K under all osmotic conditions (Fig. 2B), confirming the presence of functional KCNQ2/3 channels and that the lack of activation by cell swelling is probably not due to maximal activation of the current under isoosmotic conditions. Following expression in *Xenopus* oocytes, KCNQ2/3 channel activity was not sensitive neither to cell swelling nor to cell shrinkage [13]. One possible explanation for this apparent discrepancy could be differences in the degree of cell shrinkage between *Xenopus* oocytes and mammalian cells, another could be other differences in expression system and thereby in possible signalling pathways involved in channel inhibition during osmotic cell shrinkage.

The observation that KCNQ4 channels are activated by cell swelling in whole-cell patch-clamp experiments does not per se demonstrate that these channels are involved in the RVD process. To verify that KCNQ4 channels are activated by cell swelling in intact cells and contribute to volume recovery, cell volume changes following changes in extracellular osmolarity were measured using large angle light scattering (Fig. 3). As seen from Fig. 3A, exposing the cells to a hypoosmotic solution resulted in fast cell swelling followed by a period of RVD. Upon return to isoosmotic

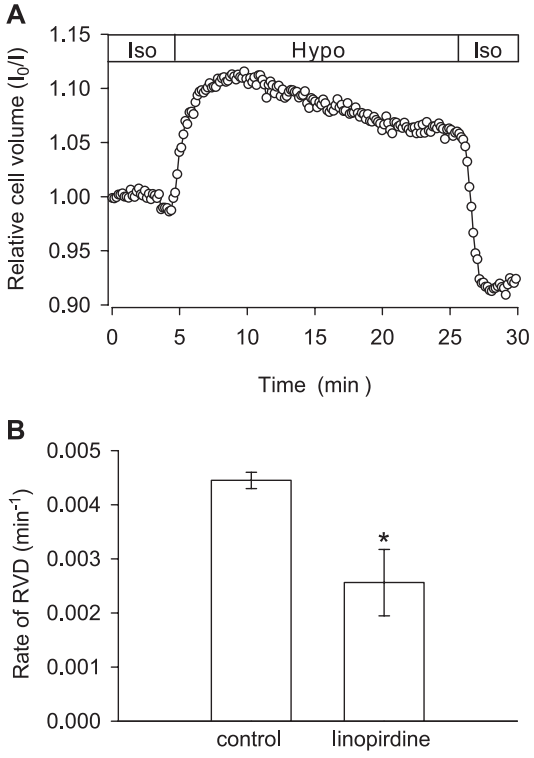

Fig. 3. KCNQ4-expressing HEK 293 cells exhibit linopirdine-sensitive RVD. (A) Cell volume changes as a function of time. Relative cell volume was measured using large-angle light scattering as described in Ref. [9]. The isoosmotic solution (300 mOsm) contained (in mM): 133 NaCl, 5 KCl, 1 $CaCl_2$, 1 Na_2HPO_4, 1 $MgSO_4$, 0.5 NaH_2PO_4, 10 HEPES, 5 glucose, pH 7.4. In the hypoosmotic solution (200 mOsm), NaCl was reduced to 85 mM. (B) Effect of linopirdine on the RVD rate in KCNQ4-expressing HEK 293 cells. RVD rates were calculated as the slope of the relative cell volume traces in the initial 300 s following maximal cell swelling in the absence (control, $n=5$) or presence of 100 μM linopirdine ($n=4$) in the hypoosmotic solution. (*) Indicates statistic different from control ($P<0.05$, Student's *t*-test for unpaired observations).

medium cell volume decreased below the initial value demonstrating that osmotic active substances have been lost from the cells. To verify the contribution of KCNQ4 channels to RVD, cells were exposed to the hypoosmotic solution in the absence (control) or presence of the KCNQ channel blocker, linopirdine (Fig. 3B). As seen, linopirdine significantly inhibited the RVD rate in KCNQ4-expressing HEK 293 cells, demonstrating that in the intact cell system, KCNQ4 channels are activated by cell swelling and contribute to the RVD process. The residual RVD observed in the presence of linopirdine could perhaps be due to KCl loss via a KCl co-transport mechanism, which is unlikely to be affected by this channel blocker.

Modulation of KCNQ4 channels by cellular signalling pathways is not well described. Here we investigated the possible role of classical pathways such as PKC, PKA, tyrosine phosphorylation, G proteins and Ca^{2+} as modulators of KCNQ4 activity both under isoosmotic and hypoosmotic conditions (Fig. 4). Interference with these pathways has prominent effects on RVD and osmolyte loss following swelling in numerous cell types, for review see, e.g. Refs. [19,20]. Activation of PKC by short-term exposure to PMA significantly inhibited the KCNQ4 current under isoosmotic conditions, but was without effect on the potentiation of the current by cell swelling (Fig. 4A). Activation of PKA by 8-Br-cAMP stimulated, whereas inhibition of PKA by Rp-cAMP was without effect on the isoosmotic KCNQ4 current, demonstrating that PKA activation stimulates KCNQ4 channels but is not required for normal KCNQ4 activity (Fig. 4B). Neither stimulation nor inhibition of PKA affected the hypoosmotic potentiation of the KCNQ4 current (Fig. 4B). In agreement with these observations, PKC was found to inhibit and PKA to stimulate the activity of the closely related KCNQ1 channel under isoosmotic conditions but to be without effect on the activation of these channel by increases in cell volume [13].

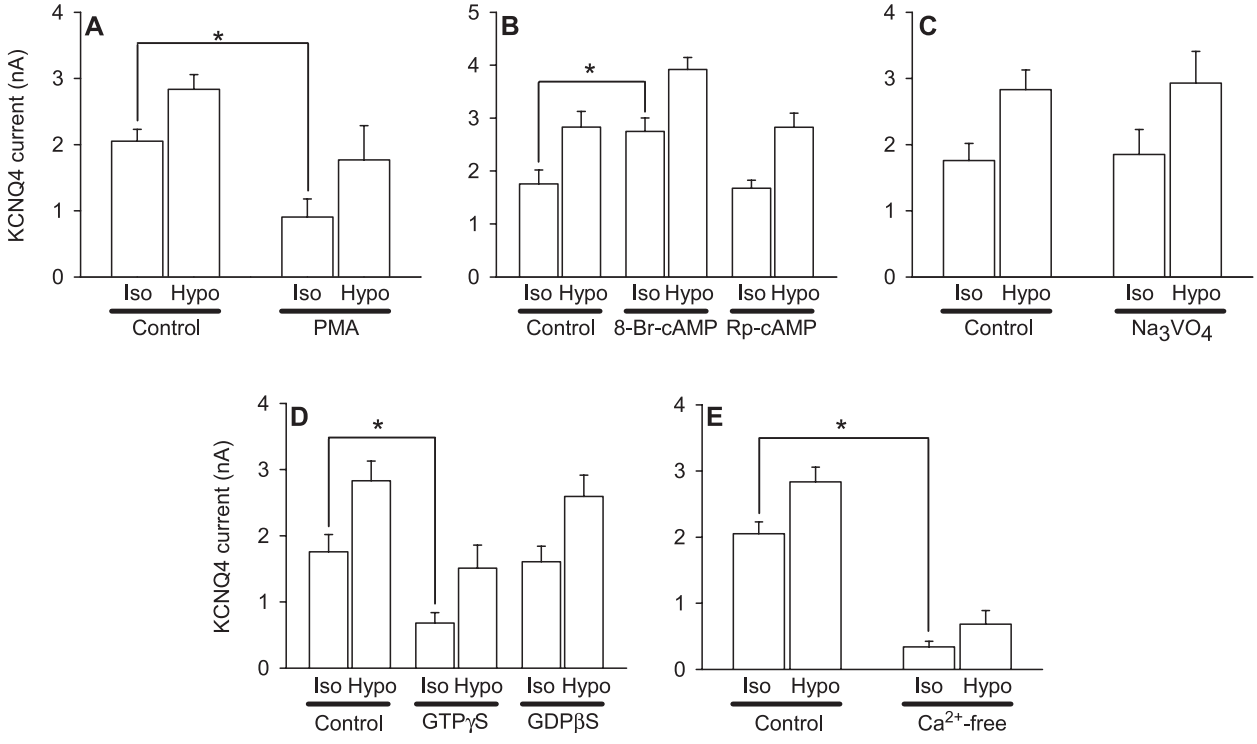

Fig. 4. Modulation of the KCNQ4 current by cellular signalling pathways. In all experiments, the KCNQ4 current magnitude was measured at 0 mV under isoosmotic conditions (Iso) and 90 s after hypoosmotic cell swelling (Hypo). (A) The role of PKC in modulation of KCNQ4 activity. PMA (100 nM) was added to the perfusion solution after obtaining a stable current level under isoosmotic conditions ($n=6$). Control experiments ($n=16$) were performed in a similar manner but in the absence of PMA. (B) PKA as a modulator of KCNQ4 channels. 8-Br-cAMP (1 mM, $n=9$) or Rp-cAMP (1 mM, $n=5$) was added to the pipette solution. Experiments were initiated 8 min after establishing the whole-cell configuration to allow adequate equilibration between the pipette solution and the intracellular compartment. Control experiments ($n=10$) were performed in a similar manner but in the absence of 8-Br-cAMP and Rp-cAMP. (C) Role of tyrosine phosphorylation in modulation of KCNQ4 channels. Experiments were conducted as in B, but Na_3VO_4 (400 μM, $n=5$) was added to the pipette solution. (D) Role of G-proteins in the modulation of KCNQ4 channels. Experiments were conducted as in B except that GTPγS (40 μM, $n=7$) or GDPβ (1 mM, $n=6$) was added to the pipette solution. (E) Role of Ca^{2+} in the modulation of KCNQ4 channels. Experiments were performed at a "permissive" Ca^{2+} level (0.1 mM EGTA, $n=16$, Control) or in the absence of Ca^{2+} (10 mM EGTA, $n=7$). (*) Indicates significant difference ($P<0.05$, Student's t-test for unpaired experiments). Please note that the current during hypoosmotic exposure in all experiments was significantly higher than under isoosmotic conditions ($P<0.05$, paired observations). The swelling-induced increase in current was not significantly different in treated and untreated cells, except in experiments performed in the absence of Ca^{2+} ($P<0.05$, unpaired experiments). However, in the absence of Ca^{2+}, the relative swelling-induced increase was not reduced (please see text for details).

It has been suggested that one of the early signalling events following cell swelling is the activation of a tyrosine kinase. RVD is blunted by inhibitors of tyrosine kinases, see Ref. [21] and protein tyrosine phosphorylation is involved in the regulation of, e.g. the volume-sensitive Cl^- channels [22,23], the volume-sensitive taurine efflux pathway [24,25] and the KCl co-transporter [26]. The Src tyrosine kinase decreased the KCNQ4 current amplitude when it was overexpressed together with the channels in Chinese hamster ovary cells [27], suggesting that a tyrosine kinase(s) could play a central role in the regulation of KCNQ4 channel activity. Increasing cellular tyrosine phosphorylation by application of the tyrosine phosphatase inhibitor Na_3VO_4 increased the amplitude of the volume-sensitive Cl^- current in the HEK 293 cells ($n=5$, not shown), similar to previous reports from other cell lines [22,23] but was without affect on both the isoosmotic and the hypoosmotic KCNQ4 current (Fig. 4C). In addition, the specific src kinase inhibitor PP2 (400 nM, $n=3$) had no significant affect on the isoosmotic KCNQ4 current and likewise failed to affect the potentiation of the current by cell swelling. The lack of effect of PP2 on the KCNQ4 current found in the present study might be explained by low endogenous src-like activity in HEK 293 cells. However, taken together, the results obtained in the present study argue against a significant role of tyrosine phosphorylation events in the modulation of KCNQ4 channels by changes in cell volume. Both monomeric and heterotrimeric G proteins seem to be involved in the RVD response, and addition of GTPγS, a compound known to activate G proteins, has been shown to accelerate the RVD response [28,29] and to activate a Cl^- conductance with similar properties to the one activated by cell swelling [23]. Activation of G proteins by GTPγS resulted in a reduction in the KCNQ4 current amplitude under isoosmotic conditions (Fig. 4D), but failed to affect the potentiation of the current by cell swelling. The inhibitory effect of GTPγS under isoosmotic conditions is in agreement with several reports demonstrating that M-currents are inhibited following activation of G protein coupled receptors like, e.g. muscarinic, adrenergic and purinergic receptors, see Ref. [30]. In a recent report, GTPγS was found to stimulate the KCNQ4-mediated current when the channels were expressed in *Xenopus* oocytes [31]. The reason for this apparent discrepancy is unclear, but might be explained by differences in the experimental conditions; in this context *Xenopus* oocytes are rather promiscuous with respect to G protein coupling and signal transduction pathways. Inhibition of G proteins by GDPβS was without effect on both iso- and hypoosmotic KCNQ4 current activity (Fig. 4D) and taken together the results point against an important role for G proteins in the swelling-induced activation of KCNQ4 channels. It should be noted that GTPγS produced a transient increase in the Cl^- current in HEK 293 cells under isoosmotic conditions (not shown) in agreement with previous reports in CPAE cells [23]. Finally, we investigated whether there was a requirement for Ca^{2+} in the activation of the KCNQ4 channels by omitting Ca^{2+} from the extracellular solution and buffering intracellular Ca^{2+} by high concentrations of EGTA (Fig. 4E). Removing Ca^{2+} strongly suppressed the KCNQ4 current under both iso- and hypoosmotic conditions. However, even in the absence of Ca^{2+}, cell swelling still produced a significant increase in the KCNQ4 current amplitude demonstrating that Ca^{2+} is not the primary messenger responsible for activation of these channels after swelling. At permissive Ca^{2+} concentrations the KCNQ4 current during cell swelling was 1.40 ± 0.03 ($n=16$) relative to isoosmotic control, in the absence of Ca^{2+} the swelling-induced KCNQ4 current was 1.90 ± 0.16 ($n=7$) relative to isoosmotic control. The role of Ca^{2+} in regulation of native M-current activity seems somewhat obscure ranging from inhibiting [32], no role [33] to stimulation [34]. From our experiments, it is clear that a certain Ca^{2+} level is required for normal KCNQ4 activity following expression in HEK 293 cells. It is, however, not clear whether the requirement for Ca^{2+} is directly related to the channel, or due to effects on intracellular signalling pathways.

In conclusion, we find that KCNQ4 channels expressed in HEK 293 cells are sensitive to cell volume changes, being stimulated by cell swelling and inhibited by cell shrinkage, respectively. Unlike the KCNQ4 channels, KCNQ2/3 heteromeric channels are not activated by cell swelling, but this channel complex can still be inhibited by cell shrinkage. Besides being activated by cell swelling in patch-clamp experiments, KCNQ4 channels also contribute to the RVD process in intact cells. Under isoosmotic conditions PKA, PKC, G proteins and Ca^{2+} can modulate the KCNQ4 channels whereas protein tyrosine phosphorylation only plays a minor role in KCNQ4 channel modulation. The potentiation of the KCNQ4 current by cell swelling is unaffected by PKA, PKC, G proteins and protein tyrosine phosphorylation. A certain Ca^{2+} level may be required for full KCNQ4 activity after swelling but Ca^{2+} is not the primary messenger responsible for the increase in KCNQ4 current induced by cell swelling.

Acknowledgements

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