Journal of Cell Science

Endoplasmic reticulum potassium–hydrogen exchanger and small conductance calcium-activated potassium channel activities are essential for ER calcium uptake in neurons and cardiomyocytes

Malle Kuum[1,2,3], Vladimir Veksler[2,3], Joanna Liiv[1], Renee Ventura-Clapier[2,3] and Allen Kaasik[1,*]

[1]Department of Pharmacology, Centre of Excellence for Translational Medicine, University of Tartu, Ravila 19, Tartu EE-51014, Estonia
[2]INSERM, U-769, 5, rue Jean-Baptiste Clement, Châtenay-Malabry F-92296, France
[3]Université Paris-Sud, 5, rue Jean-Baptiste Clement, Châtenay-Malabry F-92296, France
*Author for correspondence (allen.kaasik@ut.ee)

Accepted 12 September 2011
Journal of Cell Science 125, 625–633
© 2012. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.090126

Summary

Calcium pumping into the endoplasmic reticulum (ER) lumen is thought to be coupled to a countertransport of protons through sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and the members of the ClC family of chloride channels. However, pH in the ER lumen remains neutral, which suggests a mechanism responsible for proton re-entry. We studied whether cation–proton exchangers could act as routes for such a re-entry. ER Ca^{2+} uptake was measured in permeabilized immortalized hypothalamic neurons, primary rat cortical neurons and mouse cardiac fibers. Replacement of K^+ in the uptake solution with Na^+ or tetraethylammonium led to a strong inhibition of Ca^{2+} uptake in neurons and cardiomyocytes. Furthermore, inhibitors of the potassium–proton exchanger (quinine or propranolol) but not of the sodium–proton exchanger reduced ER Ca^{2+} uptake by 56–82%. Externally added nigericin, a potassium–proton exchanger, attenuated the inhibitory effect of propranolol. Inhibitors of small conductance calcium-sensitive K^+ (SK_{Ca}) channels (UCL 1684, dequalinium) blocked the uptake of Ca^{2+} by the ER in all preparations by 48–94%, whereas inhibitors of other K^+ channels (IK_{Ca}, BK_{Ca} and K_{ATP}) had no effect. Fluorescence microscopy and western blot analysis revealed the presence of both SK_{Ca} channels and the potassium–proton exchanger leucine zipper-EF-hand-containing transmembrane protein 1 (LETM1) in ER in situ and in the purified ER fraction. The data obtained demonstrate that SK_{Ca} channels and LETM1 reside in the ER membrane and that their activity is essential for ER Ca^{2+} uptake.

Key words: SK_{Ca} channels, LETM1, Endoplasmic reticulum, Counterion fluxes

Introduction

The endoplasmic reticulum (ER), also called sarcoplasmic reticulum (SR) in muscle cells, serves as a dynamic Ca^{2+} pool involved in rapid signaling events associated with cell stimulation in number of cell types. This function is supported by ATP-dependent Ca^{2+} pumps residing in the ER membrane (SERCAs: sarcoplasmic/endoplasmic reticulum calcium ATPases). In the heart, Ca^{2+} is cyclically released into the cytoplasm when cardiac systole begins and is then recaptured into the lumen of the SR, predominantly by the SERCA2a isoform. In the brain, Ca^{2+} is released from the ER during neuronal firing and is again taken up by the SERCA2b isoform during the repolarization phase.

It is well known that SERCA activity is regulated by Ca^{2+} as well as by some ER membrane proteins (phospholamban or sarcolipin). However, there is another important regulatory factor, namely, counterion movement. Indeed, Ca^{2+} transport into the ER lumen is electrically neutral and thus should be accompanied by cation export.

Previous studies have demonstrated that the transfer of Ca^{2+} to the ER lumen is coupled to a countertransport of protons (Levy et al., 1990; Ueno and Sekine, 1981; Yu et al., 1993). For two Ca^{2+} ions, the countertransport of two or possibly three protons from the ER lumen to cytoplasm, maintains, more or less, the electroneutrality across the ER membrane (Levy et al., 1990).

Theoretically, the electroneutrality could also be achieved by ER transmembrane fluxes of ions that are abundant in the cell, particularly K^+, but also Na^+ or Cl^-. It has been proposed that the remaining intraluminal positive charges could be balanced by Cl^- influx into the ER lumen (Edwards and Kahl, 2010; Hirota et al., 2006; Pollock et al., 1998). Chloride-channel (ClC)-type proteins are present in the ER and colocalize with SERCA. Recent evidence suggests also that intracellular ClC3–7 proteins are exchangers rather than channels (Jentsch, 2007; Picollo and Pusch, 2005; Plans et al., 2009; Scheel et al., 2005). The stoichiometry of ion exchange is thought to be approximately 2 Cl^- for 1 H^+ for ClC4, 5 and 7 (Graves et al., 2008; Picollo and Pusch, 2005; Scheel et al., 2005). In spite of relatively massive Ca^{2+}- and Cl^--induced proton efflux, pH in the ER lumen remains neutral (Kim et al., 1998; Kneen et al., 1998), which suggests a mechanism (through channels or ion exchangers) that is responsible for proton re-entry. The existence of such channels and/or exchangers has the utmost importance because ER lumen alkalinization would lead to a considerable inhibition of SERCA

activity because the Ca^{2+}-pumping activity of SERCA has been reported to stop at approximately luminal pH 8.0 (Peinelt and Apell, 2002). One of the candidates for such a function could be a potassium–hydrogen exchanger (KHE) such as that identified in the mitochondrial inner membrane (Froschauer et al., 2005; McQuibban et al., 2010; Nowikovsky et al., 2004). To our knowledge, these exchangers have not been identified in the ER membrane; however, one of the isoforms has a signal peptide for the ER membrane. The relevance of a sodium–hydrogen exchanger (NHE) also cannot be excluded as at least one of the NHE proteins (NHE6) also has a signal peptide for the ER membrane (Miyazaki et al., 2001). Involvement of KHE and/or NHEs means that cations should be extruded from the ER during uptake of Ca^{2+} by the ER, and the ER might also need additional pathways for influx of these ions. Indeed, both ATP-sensitive potassium (K_{ATP}) channels and some Ca^{2+}-activated potassium channels [big conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channels] have been shown to reside in the ER membrane (Zhou et al., 2005; Yamashita et al., 2006).

Thus, the present study was designed to investigate the possible role of cation fluxes in ER Ca^{2+} uptake. We used permeabilized preparations of neurons and cardiac fibers (e.g. cells in which ER Ca^{2+} fluxes are thought to play the highest role), to investigate the ER function in situ, in its natural environment. In particular, permeabilization of plasma membrane with detergents made it possible to study the total intact ER population while maintaining the cellular architecture and controlling the intracellular milieu. Plasma membrane permeabilization by saponin is widely used for functional studies of endo(sarco)plasmic reticulum in various tissues and cells (Kargacin and Kargacin, 1995; Koch et al., 1987; Minajeva et al., 1996). At a relatively low concentration (50 µg/ml), saponin destroys the integrity of the plasma membrane, but a fivefold higher concentration is required to permeabilize the ER (Wassler et al., 1987). This difference in sensitivity to saponin can be accounted for by the different cholesterol contents of the membranes of these organelles.

Results

ER transmembrane K^+ fluxes affect ER Ca^{2+} uptake

ER Ca^{2+} uptake was measured in three different preparations using two independent methods: (1) saponin-permeabilized immortalized hypothalamic neurons (GT1-7 cells) and primary cortical neurons using Mag-fluo-4 AM fluorescent indicator; and (2) permeabilized cardiac fibers using the caffeine-induced Ca^{2+} release method. To study the relevance of K^+ flux for Ca^{2+} uptake, we first completely replaced K^+ in the uptake solution with equimolar Na^+ or with tetraethylammonium ions (TEA), known to non-selectively block K^+ channels. Both replacements led to a strong inhibition of Ca^{2+} uptake in GT1-7 cells and in primary cortical neurons (Fig. 1). Replacement of K^+ with TEA also inhibited SR Ca^{2+} uptake in permeabilized cardiac fibers (Fig. 1E). We can thus conclude that K^+ is essential for ER Ca^{2+} uptake.

Small conductance Ca^{2+}-sensitive K^+ (SK_{Ca}) channels are involved in ER Ca^{2+} uptake

In order to find out which types of K^+ channel are required for proper ER Ca^{2+} uptake, effects of different channel inhibitors on ER Ca^{2+} uptake were tested. Inhibitors of K_{ATP} channels, 5-hydroxydecanoate (5-HD; 100 µM) and glimepiride (50 µM) failed to demonstrate any effect on ER Ca^{2+} uptake in GT1-7 neurons (Fig. 2A,B), primary cortical neurons (supplementary material Fig. S2) or cardiac fibers (data not shown). Also no effect was observed when activators of K_{ATP} channels, pinacidil (200 µM) or diazoxide (150 µM) were used (Fig. 2C,D). However, as depicted in Fig. 3, inhibitors of SK_{Ca} channels, UCL 1684 (50 µM) and dequalinium (100 µM) inhibited ER

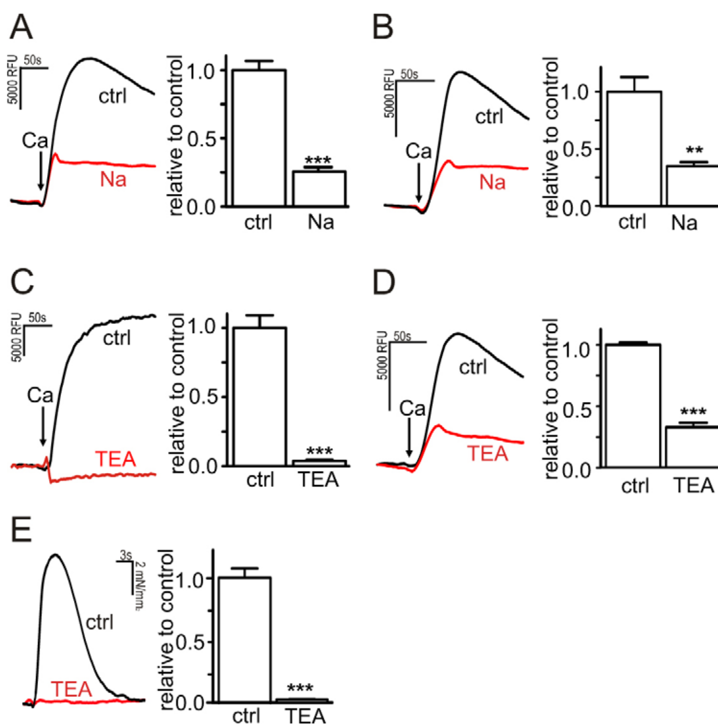

Fig. 1. Replacement of K^+ ions with Na^+ or TEA^+ inhibits in situ ER Ca^{2+} uptake. (**A–D**) Time course of luminal Ca^{2+}-dependent fluorescence after Ca^{2+} (100 nM) addition (left panels) and mean values of maximal fluorescence (right panels) in GT1-7 cells (A,C) or in primary cortical neurons (B,D). Physiological K^+ (ctrl) was replaced with equimolar Na^+ (A,B) or TEA^+ (C,D). (**E**) Tension transients elicited by 5 mM caffeine in ventricular permeabilized fibers after 5 minutes of SR Ca^{2+} loading in the presence of K^+ or TEA^+ (left panel) and mean values of tension–time integrals (right panel). Here and in subsequent figures $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control conditions (taken as 1).

Journal of Cell Science

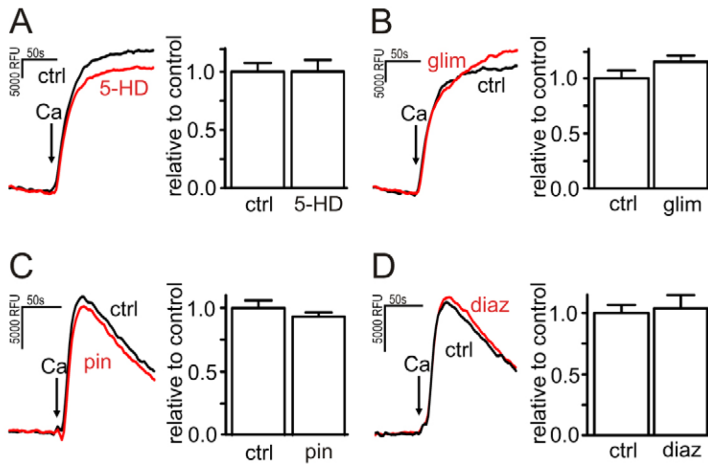

Fig. 2. Inhibitors of K_{ATP} channels as well as K_{ATP} channel openers have no effect on ER Ca^{2+} uptake in GT1-7 neurons. Time course of luminal Ca^{2+}-dependent fluorescence after Ca^{2+} addition (left panels) and mean values of maximal fluorescence (right panels) in the presence of K_{ATP} channel inhibitors 5-HD (100 µM; **A**) and glimepiride (glim, 50 µM; **B**), and K_{ATP} channel openers pinacidil (pin, 200 µM; **C**) and diazoxide (diaz, 150 µM; **D**).

Ca^{2+} uptake in GT1-7 cells (Fig. 3A,D) and primary cortical neurons (Fig. 3B,E) as well as in cardiac fiber preparations (Fig. 3C,F). Charybdotoxin (200 nM), an inhibitor of BK_{Ca} channels and TRAM 34 (10 µM), an inhibitor of intermediate conductance Ca^{2+}-sensitive K^+ (IK_{Ca}) channels, had no inhibitory effect in GT1-7 neurons (supplementary material Fig. S3A,B). Similarly, activators of SK_{Ca} channels, DC-EBIO (50 µM) and NS309 (50 µM), had no strong effect on Ca^{2+} uptake (supplementary material Fig. S3C,D). These data suggest that SK_{Ca} but not K_{ATP} channels control the K^+ fluxes required for efficient ER Ca^{2+}uptake.

To ensure the possibility that the effect of SK_{Ca} channel inhibitors on Ca^{2+} uptake is not related to direct SERCA inhibition, we measured the effect of these compounds on backward activity of SERCA. We have previously demonstrated (Kuum et al., 2009) that fully loaded SR loses Ca^{2+} by backward flux through SERCA. Under these circumstances, inhibition of SERCA after Ca^{2+} uptake will keep a high SR Ca^{2+} level by blocking Ca^{2+} leak from the SR. Indeed the SERCA inhibitor 2,5-di-tert-butylhydroquinone (BHQ) inhibited the Ca^{2+} leak from ER (i.e. backward flux through SERCA; supplementary material Fig. S4). Our results show that UCL 1684 or dequalinium failed to inhibit backward flux through SERCA but rather potentiated it. This suggests that these compounds have no direct inhibitory effect on SERCA.

KHE is also involved in ER Ca^{2+} uptake

ER transmembrane K^+ fluxes could be governed not only by specific K^+ channels but also by the KHE. In order to elucidate the role of the exchanger, we studied the effects of the KHE inhibitor, propranolol. As Fig. 4A–C shows, propranolol (500 µM) markedly inhibited Ca^{2+} uptake in GT1-7 cells, cortical neurons and cardiac fibers. Note that propranolol did not have a direct inhibitory effect on SERCA because it did not slow down backward Ca^{2+} leak through SERCA (supplementary material Fig. S4D). The role of K^+/H^+ exchange was confirmed in cardiac fibers by the external addition of the KHE, nigericin (30 µM), which was able to attenuate the inhibitory effect of propranolol (Fig. 4D). Another KHE inhibitor, quinine (500 µM), had an even stronger inhibitory effect on Ca^{2+} uptake in neurons (Fig. 4E). This could be related to the fact that quinine inhibits not only KHE but

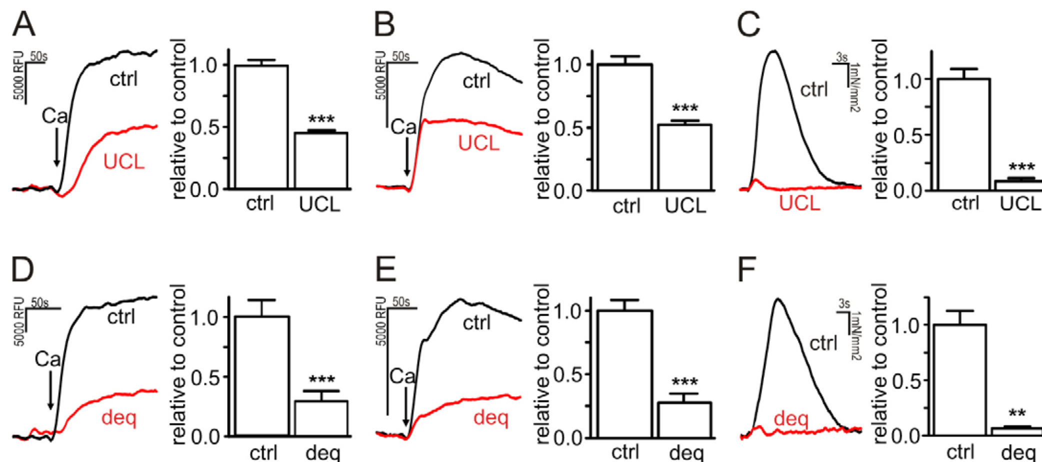

Fig. 3. Inhibitors of SK_{Ca} channels, UCL 1684 and dequalinium inhibit ER Ca^{2+} uptake. (**A**,**B**,**D**,**E**) Time course of luminal Ca^{2+}-dependent fluorescence after Ca^{2+} addition (left panels) and mean values of maximal fluorescence (right panels) in GT1-7 neurons (A,D) or in primary cortical neurons (B,E) in the presence of UCL 1684 (50 µM; A,B) or dequalinium (deq, 100 µM; D,E) as compared with control conditions (ctrl). (**C**,**F**) Force transients in ventricular permeabilized fibers elicited by 5 mM caffeine after 5 minutes of SR Ca^{2+} loading in the presence of UCL 1684 (C) or dequalinium (F; left panels) and mean values of tension–time integrals (right panels).

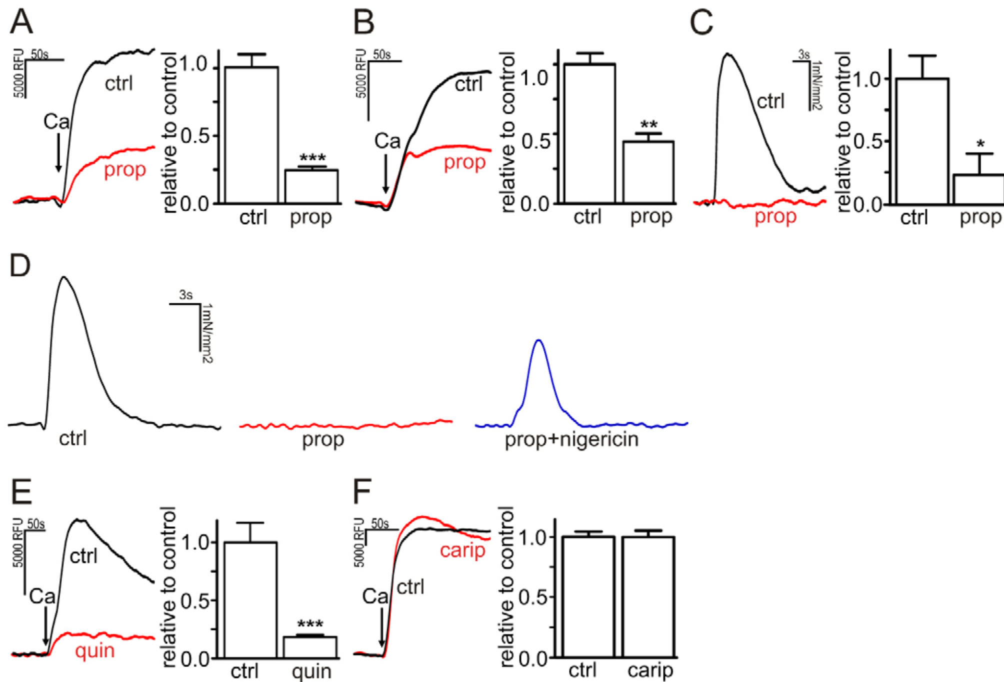

Fig. 4. Effects of KHE and NHE inhibitors on ER Ca^{2+} uptake in neurons, and force transients in ventricular permeabilized fibers elicited by caffeine after SR Ca^{2+} loading. (**A–C**) Propranolol (prop, 500 µM) inhibited ER Ca^{2+} uptake in GT1-7 cells (A), primary cortical neurons (B) and cardiac fibers (C). (**D**) Inhibitory effects of propranolol on Ca^{2+} uptake in cardiac fibers was opposed by 30 µM nigericin. (**E**,**F**) Quinine (quin, 500 µM; E) but not cariporide (carip, 50 µM; F) had an inhibitory effect on ER Ca^{2+} uptake in GT1-7 neurons.

also Ca^{2+}-dependent K^+ channels (Mancilla and Rojas, 1990), thus potentiating the inhibitory effect on ER Ca^{2+} uptake. In addition, we tested the effects of cariporide (50 µM), an inhibitor of NHEs. This drug had no effect on Ca^{2+} uptake (Fig. 4F), thus suggesting that Na^{2+}/H^+ exchange is not involved in counterion balance in the ER.

SK_{Ca} channels and KHE are expressed in ER

The above-mentioned results show the pharmacological effects of K^+ channel and KHE modulators. However, the presence of these transporters in ER membranes is still to be demonstrated. In the next series of experiments, SK_{Ca} and K_{ATP} channels were visualized using a specific antibody or glibenclamide-BODIPY FL in cortical neurons expressing the fluorescent ER marker pDsRed2-ER. In neurons, the ER is dispersed throughout the entire cytoplasm, thus complicating the analysis. We therefore examined confocal sections through the nuclei and visualized fingerlike invaginations of the nuclear membrane that were continuous with the ER and also contained ER proteins. Fig. 5A,B demonstrates that the ER–nuclear membrane contains both SK_{Ca} and K_{ATP} channels. SK_{Ca} and K_{ATP} channels were also found in cortical neurons expressing the fluorescent mitochondrial marker, pDsRed2-mito. Fig. 6 demonstrates that the pattern of mitochondrial staining was clearly different from the staining of SK_{Ca} and K_{ATP} channels, excluding the possibility that the observed intracellular signals coming from the channels could be only of mitochondrial origin.

It has been shown that mitochondrial KHE is encoded by the leucine zipper-EF-hand-containing transmembrane protein 1 (LETM1) gene (Nowikovsky et al., 2004). LETM1 has an N-terminal mitochondrial-targeting sequence and also an ER membrane sequence site in its C-terminus. We therefore examined the localization of LETM1 using a specific antibody. As Fig. 5C demonstrates, in neurons LETM1 was located in the intracellular space and colocalized with the ER. Again, the pattern of mitochondrial staining was clearly different from that of LETM1 staining, thus excluding the possibility that the observed intracellular signal could be only of mitochondrial origin (Fig. 6). Nevertheless, using antigen-retrieval techniques

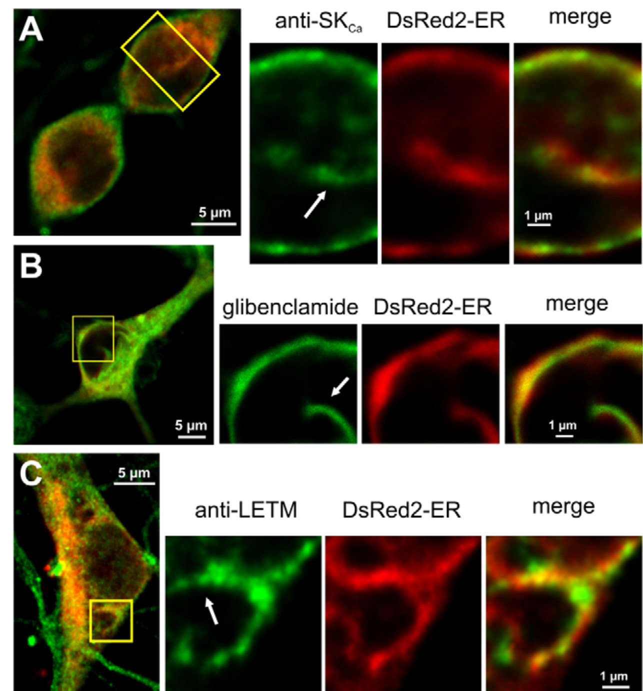

Fig. 5. SK_{Ca}, K_{ATP} channels and LETM1 colocalize with ER in cortical neurons. Antibody staining of SK_{Ca} (**A**), K_{ATP} staining (glibenclamide-BODIPY FL, **B**) and antibody staining of LETM1 (**C**) partially overlap with the ER marker pDsRed2-ER. Left panels show dispersed pattern of signals. Higher magnification images (right panels) of the boxed regions show that signals from the channels and LETM1 clearly colocalize with the ER marker. Arrows indicate finger-like invaginations of the ER–nuclear membrane.

Journal of Cell Science

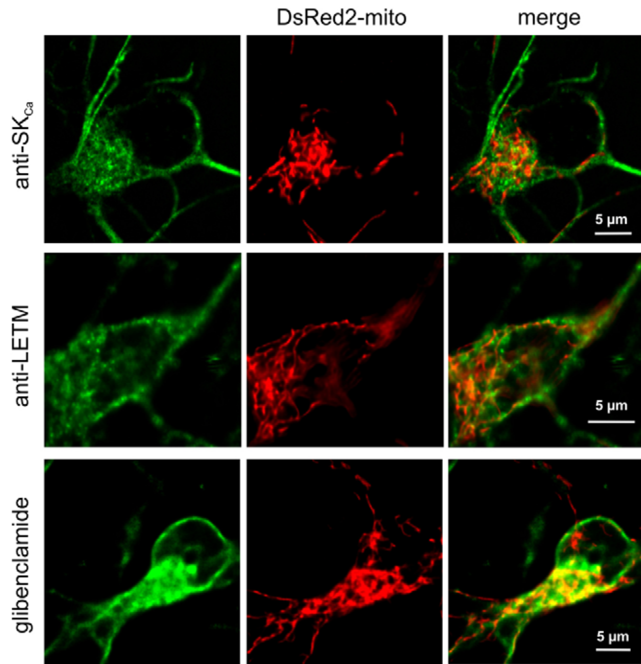

Fig. 6. SK_{Ca} and K_{ATP} channels and LETM1 visualization in cortical neurons expressing a fluorescent mitochondrial marker (DsRed2-mito). Staining of SK_{Ca} and LETM1 with antibodies and K_{ATP} with glibenclamide-BODIPY FL shows that these proteins are not restricted to mitochondria.

we were able to unmask the mitochondrial LETM1 (data not shown).

Fig. 7 compares distribution patterns of K_{ATP} channels, LETM1 and SK_{Ca} channels in cardiomyocytes with patterns of mitochondria, T-tubules and SR, visualized by Mitotracker Green, DiOC16 and anti-SERCA2 antibody (Fig. 7A–E), respectively. The distribution of ATP-dependent channels, revealed with fluorescent glibenclamide, was similar to that of the mitochondria (Fig. 7F,G), arranged in repeated rows along with myofibrils. The distribution pattern of LETM1 (Fig. 7H,I), revealed by antibody staining was clearly similar to the arrangement of the SR, with sarcomere-like repeated stripes without longitudinal elements, which are typical for T-tubules. Finally, the pattern of SK_{Ca} channels visualized by antibody staining (Fig. 7J,K), was also similar to the SR pattern, although some blurring of the signal occurred in regions occupied by mitochondria.

We also performed western blot analysis of the enriched ER fraction from brain (Fig. 8). The anti-SK_{Ca} antibody detected 65-kDa and 75-kDa proteins (lane 1) corresponding to the predicted molecular mass of the protein (64 kDa). Anti-LETM1 antibody detected 85-kDa and 170-kDa proteins (lane 2) that correspond well to the predicted molecular masses of monomeric (83-kDa) and dimeric (166-kDa) forms of the protein. The ER fraction was rich in SERCA2 (lane 3) and free of mitochondrial contamination, as confirmed by anti-cytochrome-*c*-oxidase antibody (lane 4). We also performed western blot analysis on

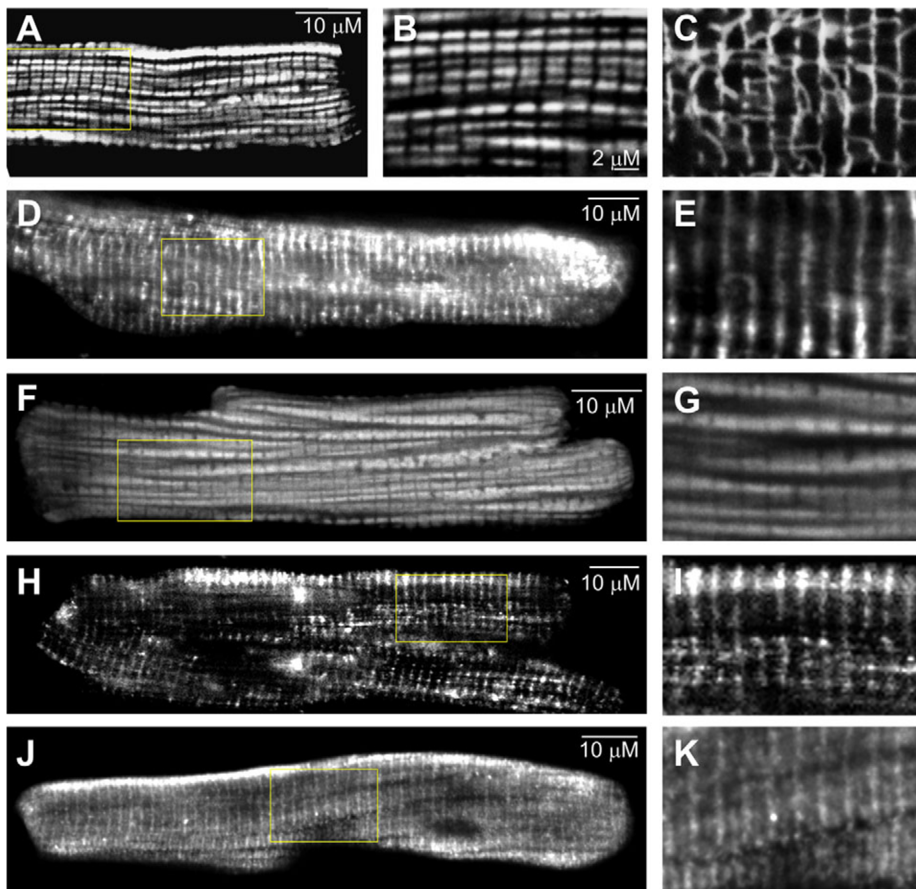

Fig. 7. Imaging of K_{ATP} channels, LETM1 and SK_{Ca} in cardiomyocytes. (**A**,**B**) Mitochondria visualized with Mitotracker Green (higher magnification in B). (**C**) T-tubules visualized with DiOC16. (**D**,**E**) SR visualized with anti-SERCA2 (higher magnification in E). (**F**,**G**) K_{ATP} signal (glibenclamide-BODIPY FL) is mostly mitochondrial (higher magnification in G). (**H**,**I**) Antibody staining of LETM1 (higher magnification in I) is the same as that of the SR. (**J**,**K**) Antibody staining of SK_{Ca} (higher magnification in K) is predominantly similar to that of the SR although some dispersed signal could be coming from mitochondria.

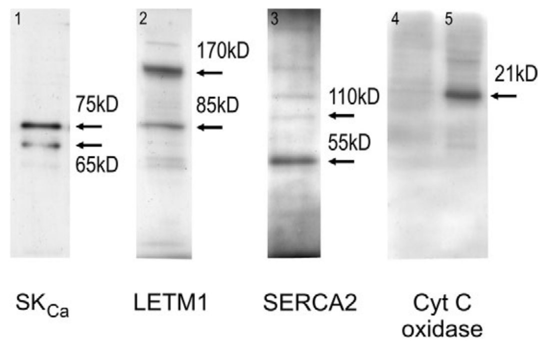

Fig. 8. Western blot analysis of the ER-enriched fraction from brain. This fraction contains SK_{Ca} (lane 1) and LETM1 (lane 2). Note that this ER fraction is rich in SERCA2 (lane 3) but is free of mitochondrial contamination of cytochrome *c* oxidase (lane 4). This mitochondrial protein is abundantly present in the mitochondria-enriched fraction (lane 5, positive control).

the mitochondrial fraction to demonstrate that anti-cytochrome-*c*-oxidase antibody indeed recognizes the protein (lane 5).

Discussion

This work is the first to demonstrate that KHE and SK_{Ca} channels are present in the ER membrane and that their activity is a prerequisite for ER Ca^{2+} uptake.

KHE has been found previously on the inner mitochondrial membrane where it allows intramitochondrial K^+ to exchange with extramitochondrial protons. We and others have shown that it is essential for controlling the mitochondrial volume and that its inhibition leads to marked mitochondrial swelling (Dimmer et al., 2008; Kaasik et al., 2004; Nowikovsky et al., 2004; Safiulina et al., 2006). KHE is encoded by the gene *LETM1* and is considered to reside only in the mitochondrial inner membrane (Froschauer et al., 2005; McQuibban et al., 2010; Nowikovsky et al., 2004). However, in addition to its N-terminal mitochondrial targeting sequence, the protein product of *LETM1* has an ER-membrane-retention signal AEVK in its C-terminus. It could be speculated that not all the LETM1 goes to mitochondria but that some is targeted also to ER membrane. Indeed, our immunohistochemical experiments with a LETM1-specific antibody demonstrate its colocalization with ER in neurons, and a distribution pattern similar to that of the ER in cardiomyocytes. Moreover, high LETM1 levels were detected in the purified ER fraction.

Our results also demonstrate that ER KHE activity is required for ER Ca^{2+} uptake. Inhibition of KHE in permeabilized neurons and cardiac fibers by propranolol or quinine markedly inhibited ER Ca^{2+} uptake. These effects could not be attributed to direct inhibition of SERCA because propranolol did not inhibit the reverse activity of SERCA, which was previously shown by us to be sensitive to SERCA inhibitors (Kuum et al., 2009). No effect was observed when the NHE inhibitor, cariporide was used, suggesting that sodium–proton exchange plays no major role in ER Ca^{2+} uptake.

What could be the physiological relevance of KHE in the ER? As noted in the Introduction, SERCA acts as an antiporter: two Ca^{2+} ions pumped into the ER lumen for two to three protons from the lumen released into the cytoplasm (Levy et al., 1990). In addition, the influx of Cl^- counterions through ClC leads to proton extrusion from the ER lumen (Jentsch, 2007; Picollo and Pusch, 2005; Plans et al., 2009; Scheel et al., 2005). Without proton re-entry, the ER lumen would be alkalinized, thus blocking further the activity of SERCA. Earlier studies have demonstrated that ER membranes are highly permeable to protons but did not identify the molecular nature of this permeability (Kim et al., 1998; Kneen et al., 1998). Our experiments strongly suggest that protons are able to re-enter the ER lumen through the KHE.

Our second important finding is that SK_{Ca} channels are located in the ER and participate in ER Ca^{2+} uptake. The SK_{Ca} channel subfamily has four members: SK1–4 (also known as KCNN1–4) (Joiner et al., 1997). Although they are thought to be located only in the plasma membrane, some of the SK_{Ca} channel proteins have ER-membrane-retention signals at their N-terminus (one of the SK1 isoforms PGPR, SK2 SSCR and one of the SK3 isoforms ERPI) that could target them to the ER membrane. However, to the best of our knowledge, there are currently no data concerning their intracellular localization and our report is the first to suggest that SK_{Ca} channels are present in the ER membrane and that they participate in ER Ca^{2+} uptake. Using an antibody recognizing SK1-3 channels we demonstrated that SK_{Ca} channels colocalized with an ER marker in neurons and that their localization resembles the distribution of SR in cardiomyocytes.

In the plasma membrane, a rise in intracellular Ca^{2+} activates the SK_{Ca} channels and leads to the outward K^+ current and hyperpolarization of the plasma membrane (Fakler and Adelman, 2008). It seems logical, therefore, that a rise in intracellular Ca^{2+} will also activate the ER SK_{Ca} channels and K^+ entry from the cytoplasm to the ER lumen that is necessary for the balance of ion charges. Our results demonstrate that two selective non-peptide SK_{Ca} channel blockers, dequalinium and UCL 1684 partially inhibited ER Ca^{2+} uptake in neurons and inhibited it completely in cardiac fibers. We did not use the widely known peptide-inhibitor apamin because its binding sites are thought to reside inside the reticular lumen (Faber, 2009) and might therefore be inaccessible to the inhibitor. Interestingly, inhibitors of BK_{Ca} channels (charybtotoxin), IK_{Ca} channels (TRAM 34) or K_{ATP} channels (5-HD and glimepiride) had no effect on ER Ca^{2+} uptake. This suggests that specifically SK_{Ca} and not other Ca^{2+}- or ATP-sensitive K^+ channels participate in the regulation of ER Ca^{2+} uptake.

The physiological role of the ER K^+ cycle can be hypothesized as follows (Fig. 9). During the Ca^{2+} uptake phase associated with proton efflux, H^+ re-entry occurs through KHE driving K^+ ions out from the lumen. Thus, the ER needs a mechanism to restore the K^+ homeostasis across the ER membrane. Our data suggest that SK_{Ca} channels provide a re-entry mechanism for K^+. Inhibitors of SK_{Ca} channel slow down Ca^{2+} uptake suggesting that activity of this channel is relevant for Ca^{2+} entering the ER. It is noteworthy that this channel is under control of cytoplasmic Ca^{2+} and we speculate that high cytoplasmic Ca^{2+} levels open ER SK_{Ca} channels, similarly to plasma-membrane-located SK_{Ca} channels that facilitate KHE function, proton re-entry and thus SERCA2 activity. However, it cannot be excluded that SK_{Ca} channels could also be used for K^+ efflux from the ER. Countertransport of protons by SERCA could not fully ensure the charge balance and further efflux of positive ions and/or influx of negative ions are required for complete charge balance. If so, then SK_{Ca} channels could also serve as a route for K^+ efflux, depending on ER Ca^{2+} content.

Journal of Cell Science

Journal of Cell Science

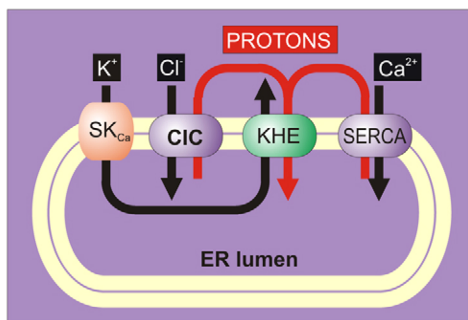

Fig. 9. Proposed model of counterion movement through the ER membrane during Ca^{2+} uptake. Ca^{2+} ions are pumped into the ER lumen by SERCA. This is accompanied by countertransport of protons (two to three protons per two Ca^{2+} ions). To compensate for the remaining positive charge, Cl^- ions enter through ClC channels that are associated with extrusion of protons (one proton per two Cl^- ions). To avoid matrix alkalization and concomitant inhibition of SERCA, protons should re-enter through KHE (or LETM1). To compensate for the loss of K^+ ions from the lumen, K^+ might re-enter through SK_{Ca} channels open only during the uptake phase.

Our results might also contribute to the understanding of Wolf–Hirschhorn syndrome (WFS). WHS is a complex congenital syndrome caused by a monoallelic deletion of the short arm of chromosome 4 (Endele et al., 1999). Variation and severity of the symptoms in WHS patients are linked to the size of the chromosome deletion and it is now widely recognized that the various disease symptoms cannot result from the loss of a single pathogenic gene. *LETM1* has been identified as an excellent candidate gene for seizures in WHS. Several studies showed that *LETM1* is deleted in most WHS patients exhibiting seizures and preserved in those without seizures (South et al., 2007; Zollino et al., 2003). To date these effects have been mostly associated with deficient mitochondrial KHE and mitochondrial osmoregulation (McQuibban et al., 2010). However, our results demonstrating participation of ER LETM1 in intracellular Ca^{2+} cycling suggest that deficient ER Ca^{2+} handling might also contribute to this pathology.

Furthermore, downregulation of the SK_{Ca} channel-mediated K^+ fluxes could potentiate epileptiform activity (Fernandez de Sevilla et al., 2006; Garduno et al., 2005). The SK_{Ca} channel enhancer 1-EBIO triggers a cessation of spontaneous oscillatory activity in hyperexcitable neuronal networks (Pedarzani et al., 2001) and epileptiform activity in hippocampal slices (Fernandez de Sevilla et al., 2006; Garduno et al., 2005). Therefore, SK_{Ca} channels have been proposed as potential new targets for the treatment of epilepsy. However, these effects might be, at least partially, related to deficient ER Ca^{2+} handling. It has been demonstrated that intracellular Ca^{2+} concentration increases during status epilepticus and that these elevations are maintained past the duration of the injury (Pal et al., 1999; Raza et al., 2004; Raza et al., 2001).

In summary, current work demonstrates that ER(SR) Ca^{2+} uptake strongly depends on transmembrane K^+ fluxes, which apparently maintain the electroneutrality across the ER(SR) membrane. These fluxes are controlled by KHE and SK_{Ca} channels, which reside in the ER(SR) membrane.

Materials and Methods

Preparation of cells and cardiac fibers

Primary cultures of rat cortical cells were prepared from neonatal Wistar rats as previously described (Wareski et al., 2009), plated onto 35 mm glass-bottom (MatTek, MA, USA) or clear-bottom microwell plates (Nunc, Roskilde, Denmark) and maintained in Neurobasal-A medium containing B-27 supplement, 2 mM GlutaMAX-I (all from Invitrogen, Carlsbad, CA, USA) and 100 μg/ml gentamicin. GT1-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM/F-12; Invitrogen) containing 10% fetal bovine serum and 100 μg/ml gentamicin. Individual rat ventricular myocytes were obtained as previously described (Verde et al., 1999). The cells were cultured in humidified 5% CO_2, 95% air at 37℃. Cardiac fibers were prepared from mouse left ventricular papillary muscles as described previously (Kuum et al., 2009). All animal experiments were performed according to the relevant regulatory standards.

Specific permeabilization of the plasmalemma was achieved by incubating the cells for 15 minutes and the fibers for 30 minutes in basic solution [which contained: 10 mM EGTA, 60 mM BES (pH 7.1), 1 mM free Mg^{2+}, 20 mM taurine, 5 mM glutamic acid, 2 mM malic acid, 3 mM K_2HPO_4, 0.5 mM dithiothreitol, 3.16 mM MgATP, 2 mM sodium azide and 12 mM Phosphocreatine, at pCa 9] that also contained 50 μg/ml saponin at 4℃. Ionic strength was adjusted to 160 mM with potassium methanesulfonate. The desired Ca^{2+} concentration was obtained by varying the CaK_2EGTA:K_2EGTA ratio.

SR Ca^{2+} uptake and leak experiments in permeabilized cardiac fibers

Fibers were tied at both ends with a natural silk thread and mounted on stainless-steel hooks between a length adjustment device and a force transducer (AE 801, Aker's Microelectronics, Horton, Norway). Fibers were immersed in 2.5 ml chambers arranged around a disc placed into a temperature-controlled bath positioned on a magnetic stirrer. All experiments were performed at 22℃ using basic solution modified as described. At the beginning of each experiment, the fiber was stretched to 120% of the slack length.

Uptake of Ca^{2+} by SR in permeabilized fibers was estimated by analyzing the tension transient caused by caffeine-induced Ca^{2+} release after SR loading. The experimental protocol used in this study was a modified version of that described previously (Minajeva et al., 1996). After emptying the SR by a brief application of caffeine (5 mM), fibers were preincubated in basic solution (at pCa 9) with or without the experimental substances for 15 minutes. SR loading was then carried out by incubation of fibers at pCa 6.5 for 5 minutes also in the presence or absence of the substances. After the loading was completed, the fibers were washed in basic solution containing no Ca^{2+} (pCa 9) for 30 seconds to remove Ca^{2+}. Excess EGTA was then washed out with basic solution containing a low concentration of EGTA (0.3 mM EGTA) for 60 seconds, SR Ca^{2+} was released with 5 mM caffeine and the mechanical response of myofibrils was recorded. For SR leak experiments, the loading was followed by 5 minutes incubation at pCa 9 in the presence or absence of substances of interest, after which Ca^{2+} was released with caffeine. The peak of caffeine-induced tension and the tension-time integral were analyzed as described previously (Minajeva et al., 1996).

ER Ca^{2+} measurement in permeabilized GT1-7 and cortical cells

A low-affinity Ca^{2+} indicator Mag-fluo-4 AM (Invitrogen) trapped within the ER was used to measure luminal free [Ca^{2+}]. Growth medium was removed and cells were incubated in Krebs solution containing 0.2 mg/ml Pluronic F127 and 5 μM Mag-fluo-4 AM (from a 5 mM stock in anhydrous DMSO) for 40 minutes at 37℃. Cells were then permeabilized in Ca^{2+}-free basic solution without substrates for mitochondria (pCa 9), containing 50 μg/ml saponin for 15 minutes at 4℃. After permeabilization, the solution was changed to basic solution with or without the experimental substance, and cells were incubated for 30 minutes at room temperature in darkness. ER Ca^{2+} measurements were performed with FlexStation™ (Molecular Devices, Sunnyvale, CA, USA) 475 nm excitation, 495 nm cut-off, 525 nm emission wavelengths at room temperature. To achieve pCa 7, Ca^{2+} was added using an automatic pipette. The observed increase in the Mag-fluo-4 signal in GT1-7 cells and cortical neurons was inhibited by the ER Ca^{2+} pump inhibitor 2,5-di-t-butyl-1,4-benzohydroquinone, BHQ (supplementary material Fig. S1A,B).

This approach was validated by confocal time-lapse imaging, which demonstrated similar increase in Mag-fluo-4 signal after addition of 100 nm Ca^{2+} (supplementary material Fig. S1). The pattern of the Mag-fluo-4 in cells resembled the ER localization.

Immunohistochemistry

Isolated cardiomyocytes were fixed with 4% paraformaldehyde in PBS for 10 minutes at 37℃. Cortical neurons were first transfected with plasmid DNA encoding pDsRed2-ER or pDsRed2-mito (Clontech Laboratories Inc., Mountain View, CA, USA) to visualize ER or mitochondria as described previously (Choubey et al., 2011) and fixed with 4% paraformaldehyde solution in growth medium in the presence of 5% sucrose for 10 minutes at 37℃. After permeabilization as described above, the cells were blocked with 10% normal goat serum at room temperature for 60 minutes and incubated with primary antibodies at 4℃ overnight. The antibodies used were: rabbit anti-LETM1 (1:250; Atlas Antibodies AB, Stockholm, Sweden), rabbit anti-potassium channel SK_{Ca} (1:200; Sigma-Aldrich, Munich, Germany), mouse anti-SERCA2 (1:500, Abcam, Cambridge, UK) and goat anti-cytochrome-*c*-oxydase-II (1:150, Santa Cruz

Biotechnology Inc., Heidelberg, Germany). After washing, the cells were further incubated with the respective fluorochrome-conjugated secondary antibodies and examined by confocal microscope using a Zeiss LSM5 Duo microscope (Carl Zeiss AG, Jena, Germany). For visualization of K_{ATP} channels, the living neurons or cardiomyocytes were stained for 15–30 minutes with 1 μM BODIPY FL glibenclamide (Invitrogen) at 37℃ before fixation.

Separation of brain ER fraction

Brain cortices from adult mice were homogenized with a Teflon–glass homogenizer in ice-cold isotonic extraction buffer containing: 10 mM HEPES (pH 7.8), 250 mM sucrose, 1 mM EGTA, 25 mM KCl, supplemented with 4% protease inhibitor cocktail (Roche, Mannheim, Germany). Nuclei and cell debris were first removed by centrifugation at 1000 ***g*** for 10 minutes at 4℃. Remaining supernatants were then further centrifuged at 12,000 ***g*** for 15 minutes 4℃ after which the mitochondria-rich pellet was collected. The remaining supernatant was centrifuged at 55,000 ***g*** for 60 minutes at 4℃ and the pellet containing the ER fraction was suspended in isotonic extraction buffer.

For western blot analysis the material was lysed in buffer containing 50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 1 mM NaF, 0.25% sodium deoxycholate and 4% protease inhibitor cocktail, for 30 minutes on ice. Equivalent amounts of total protein were separated by SDS-PAGE on 10% or 12% polyacrylamide gels and then transferred to Hybond-P PVDF transfer membranes (Amersham Biosciences, Little Chalfont, UK) in 0.1 M Tris base, 0.192 M glycine and 10% (w/w) methanol, using an electrophoretic transfer system. Further experiment was performed using SNAP i.d. Protein Detection System (Millipore, Abingdon, UK) following the manufacturer's guidelines. The membranes were blocked with 0.1% (w/w) Tween 20 in TBS containing 0.5% (w/w) non-fat dried milk at room temperature for 5 minutes. After blocking, the membranes were incubated for 15–30 minutes with primary antibodies (anti-LETM1 1:250, anti-SK_{Ca} 1:200, anti-SERCA2 1:500, anti-cytochrome-*c*-oxydase-II 1:150) followed by incubation with the appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody (1:400, Pierce, USA) for 20 minutes at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences, UK) using medical X-ray film blue (Agfa, Mortsel, Belgium). The blots probed for the proteins of interest were densitometrically analyzed using a QuantityOne 710 System (Bio-Rad, Munich, Germany).

Statistics

Results are presented as means ± s.e.m. Statistical differences were determined using *t*-tests; $P<0.05$ was accepted as significant.

Acknowledgements

We thank Ulla Peterson and Florence Lefebvre for preparation of primary cells. We also thank Valérie Domergue-Dupont and the animal core facility of IFR141 (University Paris-XI) for efficient handling and preparation of the animals.

Funding

This work was supported by the ARCHIMEDES Foundation to M.K.; the Centre National de la Recherche Scientifique to R.V.-C.; the European Regional Development Fund [grant number 3.2.0101.08-0008 to A.K.]; European Community (ESTBIOREG) [grant number grant number 205773 to A.K.]; Estonian Science Foundation [grant number 7991 to A.K.]; and the joint Estonian-French research program Parrot [grant number 25590QM to A.K. and V.V].

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.090126/-/DC1

References

Choubey, V., Safiulina, D., Vaarmann, A., Cagalinec, M., Wareski, P., Kuum, M., Zharkovsky, A. and Kaasik, A. (2011). Mutant A53T alpha-synuclein induces neuronal death by increasing mitochondrial autophagy. *J. Biol. Chem.* **286**, 10814-10824.

Dimmer, K. S., Navoni, F., Casarin, A., Trevisson, E., Endele, S., Winterpacht, A., Salviati, L. and Scorrano, L. (2008). LETM1, deleted in Wolf-Hirschhorn syndrome is required for normal mitochondrial morphology and cellular viability. *Hum. Mol. Genet.* **17**, 201-214.

Edwards, J. C. and Kahl, C. R. (2010). Chloride channels of intracellular membranes. *FEBS Lett.* **584**, 2102-2111.

Endele, S., Fuhry, M., Pak, S. J., Zabel, B. U. and Winterpacht, A. (1999). LETM1, a novel gene encoding a putative EF-hand Ca(2+)-binding protein, flanks the Wolf-Hirschhorn syndrome (WHS) critical region and is deleted in most WHS patients. *Genomics* **60**, 218-225.

Faber, E. S. (2009). Functions and modulation of neuronal SK channels. *Cell Biochem. Biophys.* **55**, 127-139.

Fakler, B. and Adelman, J. P. (2008). Control of K(Ca) channels by calcium nano/microdomains. *Neuron* **59**, 873-881.

Fernandez de Sevilla, D., Garduno, J., Galvan, E. and Buno, W. (2006). Calcium-activated afterhyperpolarizations regulate synchronization and timing of epileptiform bursts in hippocampal CA3 pyramidal neurons. *J. Neurophysiol.* **96**, 3028-3041.

Froschauer, E., Nowikovsky, K. and Schweyen, R. J. (2005). Electroneutral K^+/H^+ exchange in mitochondrial membrane vesicles involves Yol027/Letm1 proteins. *Biochim. Biophys. Acta* **1711**, 41-48.

Garduno, J., Galvan, E., Fernandez de Sevilla, D. and Buno, W. (2005). 1-Ethyl-2-benzimidazolinone (EBIO) suppresses epileptiform activity in in vitro hippocampus. *Neuropharmacology* **49**, 376-388.

Graves, A. R., Curran, P. K., Smith, C. L. and Mindell, J. A. (2008). The Cl^-/H^+ antiporter ClC-7 is the primary chloride permeation pathway in lysosomes. *Nature* **453**, 788-792.

Hirota, S., Trimble, N., Pertens, E. and Janssen, L. J. (2006). Intracellular Cl^- fluxes play a novel role in Ca^{2+} handling in airway smooth muscle. *Am. J. Physiol. Lung Cell Mol. Physiol.* **290**, L1146-L1153.

Jentsch, T. J. (2007). Chloride and the endosomal-lysosomal pathway: emerging roles of CLC chloride transporters. *J. Physiol.* **578**, 633-640.

Joiner, W. J., Wang, L. Y., Tang, M. D. and Kaczmarek, L. K. (1997). hSK4, a member of a novel subfamily of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. USA* **94**, 11013-11018.

Kaasik, A., Joubert, F., Ventura-Clapier, R. and Veksler, V. (2004). A novel mechanism of regulation of cardiac contractility by mitochondrial functional state. *FASEB J.* **18**, 1219-1227.

Kargacin, M. E. and Kargacin, G. J. (1995). Direct measurement of Ca^{2+} uptake and release by the sarcoplasmic reticulum of saponin permeabilized isolated smooth muscle cells. *J. Gen. Physiol.* **106**, 467-484.

Kim, J. H., Johannes, L., Goud, B., Antony, C., Lingwood, C. A., Daneman, R. and Grinstein, S. (1998). Noninvasive measurement of the pH of the endoplasmic reticulum at rest and during calcium release. *Proc. Natl. Acad. Sci. USA* **95**, 2997-3002.

Kneen, M., Farinas, J., Li, Y. and Verkman, A. S. (1998). Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys. J.* **74**, 1591-1599.

Koch, G. L., Macer, D. R. and Smith, M. J. (1987). Visualization of the intact endoplasmic reticulum by immunofluorescence with antibodies to the major ER glycoprotein, endoplasmin. *J. Cell Sci.* **87**, 535-542.

Kuum, M., Kaasik, A., Joubert, F., Ventura-Clapier, R. and Veksler, V. (2009). Energetic state is a strong regulator of sarcoplasmic reticulum Ca^{2+} loss in cardiac muscle: different efficiencies of different energy sources. *Cardiovasc. Res.* **83**, 89-96.

Levy, D., Seigneuret, M., Bluzat, A. and Rigaud, J. L. (1990). Evidence for proton countertransport by the sarcoplasmic reticulum Ca2(+)-ATPase during calcium transport in reconstituted proteoliposomes with low ionic permeability. *J. Biol. Chem.* **265**, 19524-19534.

Mancilla, E. and Rojas, E. (1990). Quinine blocks the high conductance, calcium-activated potassium channel in rat pancreatic beta-cells. *FEBS Lett.* **260**, 105-108.

McQuibban, A. G., Joza, N., Megighian, A., Scorzeto, M., Zanini, D., Reipert, S., Richter, C., Schweyen, R. J. and Nowikovsky, K. (2010). A Drosophila mutant of LETM1, a candidate gene for seizures in Wolf-Hirschhorn syndrome. *Hum. Mol. Genet.* **19**, 987-1000.

Minajeva, A., Ventura-Clapier, R. and Veksler, V. (1996). Ca^{2+} uptake by cardiac sarcoplasmic reticulum ATPase in situ strongly depends on bound creatine kinase. *Pflugers Arch.* **432**, 904-912.

Miyazaki, E., Sakaguchi, M., Wakabayashi, S., Shigekawa, M. and Mihara, K. (2001). NHE6 protein possesses a signal peptide destined for endoplasmic reticulum membrane and localizes in secretory organelles of the cell. *J. Biol. Chem.* **276**, 49221-49227.

Nowikovsky, K., Froschauer, E. M., Zsurka, G., Samaj, J., Reipert, S., Kolisek, M., Wiesenberger, G. and Schweyen, R. J. (2004). The LETM1/YOL027 gene family encodes a factor of the mitochondrial K+ homeostasis with a potential role in the Wolf-Hirschhorn syndrome. *J. Biol. Chem.* **279**, 30307-30315.

Pal, S., Sombati, S., Limbrick, D. D., Jr and DeLorenzo, R. J. (1999). In vitro status epilepticus causes sustained elevation of intracellular calcium levels in hippocampal neurons. *Brain Res.* **851**, 20-31.

Pedarzani, P., Mosbacher, J., Rivard, A., Cingolani, L. A., Oliver, D., Stocker, M., Adelman, J. P. and Fakler, B. (2001). Control of electrical activity in central neurons by modulating the gating of small conductance Ca2+-activated K+ channels. *J. Biol. Chem.* **276**, 9762-9769.

Peinelt, C. and Apell, H. J. (2002). Kinetics of the Ca(2+), H(+), and Mg(2+) interaction with the ion-binding sites of the SR Ca-ATPase. *Biophys. J.* **82**, 170-181.

Picollo, A. and Pusch, M. (2005). Chloride/proton antiporter activity of mammalian CLC proteins ClC-4 and ClC-5. *Nature* **436**, 420-423.

Plans, V., Rickheit, G. and Jentsch, T. J. (2009). Physiological roles of CLC Cl(-)/H(+) exchangers in renal proximal tubules. *Pflugers Arch.* **458**, 23-37.

Pollock, N. S., Kargacin, M. E. and Kargacin, G. J. (1998). Chloride channel blockers inhibit Ca^{2+} uptake by the smooth muscle sarcoplasmic reticulum. *Biophys. J.* **75**, 1759-1766.

Raza, M., Pal, S., Rafiq, A. and DeLorenzo, R. J. (2001). Long-term alteration of calcium homeostatic mechanisms in the pilocarpine model of temporal lobe epilepsy. *Brain Res.* **903**, 1-12.

Raza, M., Blair, R. E., Sombati, S., Carter, D. S., Deshpande, L. S. and DeLorenzo, R. J. (2004). Evidence that injury-induced changes in hippocampal neuronal calcium dynamics during epileptogenesis cause acquired epilepsy. *Proc. Natl. Acad. Sci. USA* **101**, 17522-17527.

Safiulina, D., Veksler, V., Zharkovsky, A. and Kaasik, A. (2006). Loss of mitochondrial membrane potential is associated with increase in mitochondrial volume: physiological role in neurones. *J. Cell. Physiol.* **206**, 347-353.

Scheel, O., Zdebik, A. A., Lourdel, S. and Jentsch, T. J. (2005). Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins. *Nature* **436**, 424-427.

South, S. T., Bleyl, S. B. and Carey, J. C. (2007). Two unique patients with novel microdeletions in 4p16.3 that exclude the WHS critical regions: implications for critical region designation. *Am. J. Med. Genet. A* **143A**, 2137-2142.

Ueno, T. and Sekine, T. (1981). A role of H+ flux in active Ca^{2+} transport into sarcoplasmic reticulum vesicles. II. H+ ejection during Ca^{2+} uptake. *J. Biochem.* **89**, 1247-1252.

Verde, I., Vandecasteele, G., Lezoualc'h, F. and Fischmeister, R. (1999). Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type Ca2+ current in rat ventricular myocytes. *Br. J. Pharmacol.* **127**, 65-74.

Wareski, P., Vaarmann, A., Choubey, V., Safiulina, D., Liiv, J., Kuum, M. and Kaasik, A. (2009). PGC-1{alpha} and PGC-1{beta} regulate mitochondrial density in neurons. *J. Biol. Chem.* **284**, 21379-21385.

Wassler, M., Jonasson, I., Persson, R. and Fries, E. (1987). Differential permeabilization of membranes by saponin treatment of isolated rat hepatocytes. Release of secretory proteins. *Biochem. J.* **247**, 407-415.

Yamashita, M., Sugioka, M. and Ogawa, Y. (2006). Voltage- and Ca^{2+}-activated potassium channels in Ca^{2+} store control Ca^{2+} release. *FEBS J.* **273**, 3585-3597.

Yu, X., Carroll, S., Rigaud, J. L. and Inesi, G. (1993). H+ countertransport and electrogenicity of the sarcoplasmic reticulum Ca^{2+} pump in reconstituted proteoliposomes. *Biophys. J.* **64**, 1232-1242.

Zhou, M., Tanaka, O., Sekiguchi, M., He, H. J., Yasuoka, Y., Itoh, H., Kawahara, K. and Abe, H. (2005). ATP-sensitive K^+-channel subunits on the mitochondria and endoplasmic reticulum of rat cardiomyocytes. *J. Histochem. Cytochem.* **53**, 1491-1500.

Zollino, M., Lecce, R., Fischetto, R., Murdolo, M., Faravelli, F., Selicorni, A., Butte, C., Memo, L., Capovilla, G. and Neri, G. (2003). Mapping the Wolf-Hirschhorn syndrome phenotype outside the currently accepted WHS critical region and defining a new critical region, WHSCR-2. *Am. J. Hum. Genet.* **72**, 590-597.