Lysosome mediated Kir2.1 breakdown directly influences inward rectifier current density

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Abstract

The inward rectifier current generated by Kir2.1 ion channel proteins is primarily responsible for the stable resting membrane potential in various excitable cell types, like neurons and myocytes. Tight regulation of Kir2.1 functioning prevents premature action potential formation and ensures optimal repolarization times. While Kir2.1 forward trafficking has been addressed in a number of studies, its degradation pathways are thus far unknown. Using three different lysosomal inhibitors, NH₄Cl, chloroquine and leupeptin, we now demonstrate involvement of the lysosomal degradation pathway in Kir2.1 breakdown. Upon application of the inhibitors, increased steady state protein levels are detectable within few hours coinciding with intracellular granular Kir2.1 accumulation. Treatment for 24 h with either chloroquine or leupeptin results in increased plasmamembrane originating inward rectifier current densities, while current–voltage characteristics remain unaltered. We conclude that the lysosomal degradation pathway contributes to Kir2.1 mediated inward rectifier current regulation.

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The formation of an action potential (AP) stands at the basis of many physiological processes. A number of distinct diseases of the cardiovascular system (atrial or ventricular fibrillation), the neuronal system (seizures, migraine) and the motor system (myotonia) can be related to ion channel malfunctioning [1]. The AP of excitable cells, such as neurons and myocytes, is the resultant of sequential and coordinated activity of a number of ion channels. Depolarizing currents are generally carried by sodium and calcium ions, while repolarizing currents result mainly from potassium fluxes. The inward rectifier potassium current ($I_{K1}$) is one of the few that operates between subsequent APs and is primarily responsible for generating and stabilizing the resting membrane potential at a rather negative level between −75 and −90 mV, and secondly for the initial depolarization (indirectly, opposing depolarizing currents) and fine tuning of final repolarization (directly, contributing repolarizing current) of the AP [2]. In mammals, several different but closely related ion channel proteins constitute the cardiac ventricular $I_{K1}$ channel. Of these, the KCNJ2 and KCNJ12 gene products Kir2.1 and Kir2.2 are the main determinants. To function as an ion channel, Kir2.x proteins form either homotypic or heterotypic tetramers defined by specific sequence domains [3]. Several studies indicate that manipulating $I_{K1}$ by means of null mutation [4], overexpression [5–7] or dominant negative expression of Kir2.1 [6,8] elucidates the importance of Kir2.1 mediated $I_{K1}$ for normal AP formation and control of sinus rhythm. The ultimate expression level of Kir2.1 may influence the eventual arrhythmogenic outcome. In humans, loss-of-function mutations in Kir2.1 lead to Andersen–Tawil syndrome which is characterized by potentially lethal ventricular arrhythmias, periodic paralysis and dysmorphic features, further emphasizing the pleiotropic action of Kir2.1 in development and adult physiology [9].

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 Trafficking of potassium channels involves a large number of subsequent steps, all of which are subject to tight regulation [10]. Several elegant studies have addressed molecular mechanisms of Kir2.x trafficking towards and anchoring at the plasma membrane. This disclosed several Kir2.x intracellular N- and C-terminal domains and interacting proteins involved in endoplasmic reticulum retention, forward trafficking and plasma membrane targeting [11–17]. Several human KCNK2 mutations that display trafficking defects have been identified [18,19], but interestingly these mutations are not in the previously mentioned export signal sequences. Finally, celastrol, a bioactive compound with strong antioxidant characteristics, inhibits Kir2.1 trafficking towards the plasmamembrane in HEK293 cells [20,21]. As demonstrated by Tong et al., tyrosine242 of Kir2.1 is involved in clathrin mediated endocytosis, by a tyrosine kinase dependent mechanism [22]. However, beyond endocytosis no experimental evidence has been described on the pathways involved in Kir2.x degradation thus far. In this study we focused on the lysosomal degradation pathway by using three different inhibitors of lysosomal breakdown [23]. The lysosomal degradation pathway starts in endosomes containing trapped membrane proteins, followed by fusion with early lysosomes which results in mature lysosomes (for reviews see [24,25]). The acid environment in the lysosomes is required for protein digestion by acidic hydrolases. We used the lysosomal protease inhibitors chloroquine, ammoniumchloride (NH₄Cl) and leupeptin. Chloroquine, an antimalarial drug, and NH₄Cl are weak bases, increasing the lysosomal pH and thereby prohibiting the breakdown of proteins by hydrolases. Furthermore, both chemicals inhibit the transport of hydrolases to the lysosomes. Leupeptin, on the other hand, acts directly as an inhibitor of the hydrolases. Interestingly, leupeptin appears to affect only the lysosomes, whereas NH₄Cl and chloroquine influence both the lysosomes and the endosomes. In this study we demonstrate that inhibition of lysosomal protein breakdown results in increased steady state level and intracellular accumulation of Kir2.1 protein, and elevated Iₖ₁ current densities.

Materials and methods

Cell culture and pharmacological treatment. HEK-KWGF cells stably expressing wildtype murine Kir2.1–GFP fusion protein were generated and cultured as described previously [26]. HEK-HsKir2.1 cells stably express non-tagged human Kir2.1 from a pCDNA3 (Invitrogen, Breda, The Netherlands) based expression vector. For lysosomal degradation no experimental evidence has been described on the pathways involved in Kir2.x degradation thus far. In this study we focused on the lysosomal degradation pathway by using three different inhibitors of lysosomal breakdown [23]. The lysosomal degradation pathway starts in endosomes containing trapped membrane proteins, followed by fusion with early lysosomes which results in mature lysosomes (for reviews see [24,25]). The acid environment in the lysosomes is required for protein digestion by acidic hydrolases. We used the lysosomal protease inhibitors chloroquine, ammoniumchloride (NH₄Cl) and leupeptin. Chloroquine, an antimalarial drug, and NH₄Cl are weak bases, increasing the lysosomal pH and thereby prohibiting the breakdown of proteins by hydrolases. Furthermore, both chemicals inhibit the transport of hydrolases to the lysosomes. Leupeptin, on the other hand, acts directly as an inhibitor of the hydrolases. Interestingly, leupeptin appears to affect only the lysosomes, whereas NH₄Cl and chloroquine influence both the lysosomes and the endosomes. In this study we demonstrate that inhibition of lysosomal protein breakdown results in increased steady state level and intracellular accumulation of Kir2.1 protein, and elevated Iₖ₁ current densities.

Inhibition of lysosomal breakdown pathways increases Kir2.1 steady state levels

HEK-KWGF cells were incubated with either 1 mM NH₄Cl, 10 μM chloroquine or 5 μg/mL leupeptin for 6 and 24 h, respectively. Subsequently, expression level of Kir2.1–GFP fusion protein was analyzed by Western blotting. As depicted in Fig. 1A, chloroquine rapidly increased Kir2.1–GFP expression levels within 6 h which was even more pronounced after 24 h. Leupeptin resulted in enhanced Kir2.1–GFP following 6 and 24 h of incubation. Only modest increased Kir2.1–GFP levels were observed due to chloroquine or leupeptin treatment, an additional signal is observed at ~70 kDa. Since the fusion protein is detected by an antibody directed against the C-terminal fused GFP, this product most likely represents a protein product that results from N-terminal Kir2.1 cleavage. To further elucidate the kinetics of Kir2.1 upregulation, HEK-KWGF cells were treated with 10 μM chloroquine and blotted onto nitrocellulose membrane (Bio-Rad, Veenendaal, The Netherlands). Blots were incubated with GFP (cat. no. Sc-9996; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Pan-Cadherin (cat. no. C1821; Sigma) primary antibodies and peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Standard ECL procedure was used as final detection (Amersham Bioscience, Buckinghamshire, UK).

Immunofluorescence microscopy. HEK-KWGF or HEK-HsKir2.1 cells cultured on Ø 11 mm glass cover slips (Smitthew, Warley, UK) were fixed using 4% paraformaldehyde. After permeabilization with 0.2% Triton X-100 (BDH) in PBS, cells were pre-incubated with 2% BSA. Next, cells were incubated overnight with either anti-GFP or anti-Kir2.1 (cat. no. Sc-18708; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody for 1 h, followed by incubation with anti-mouse or anti-goat FITC conjugated secondary antibody (Jackson ImmunoResearch) for 2 h. Cover slips were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and imaged using a Nikon Optiphot-2 microscope equipped for epifluorescence.

Electrophysiology. Iₖ₁-currents were recorded using the whole cell voltage clamp configuration in randomly chosen single HEK-KWGF cells using an Axopatch 200B amplifier (Molecular Devices, Toronto, Canada). Currents were low-pass filtered at 2 kHz and recorded at 4 kHz using a Apple PowerMac fitted with A/D card (National Instruments, Austin, TX, USA). From a holding potential of ~40 mV, 750 ms long square test pulses to potentials ranging between ~70 and +50 mV were applied to elicit membrane currents. Steady state currents at the end of the test pulse were normalized to membrane capacitance and plotted versus test potential. To obtain membrane conductances, the slope of I–V plot between ~70 and ~80 mV was determined using linear regression.

Experiments were done at 20 °C using an extracellular solution containing (mmol/L) 140 NaCl, 17.5 NaCO₃, 15 Hepes, 6 glucose, 5.4 KCl, 1.8 CaCl₂ and 1 MgCl₂ in H₂O (pH 7.20, NaOH). Patch pipettes were filled with an internal solution containing (mmol/L) 125 potassium gluconate, 10 KCl, 5 EGTA, 4 Na₂ATP, 2 MgCl₂ and 0.6 CaCl₂ in H₂O (pH 7.20, KOH) and had resistances ranging between 2 and 5 MΩ. Liquid junction potential was calculated using pClamp (Molecular Devices) and used for offline correction.

Statistics. All data are presented as mean ± SEM. Differences among groups were evaluated using one-way ANOVA and a post-hoc Holm–Sidak test, significance was assumed if p < 0.05.

Results

Inhibition of lysosomal breakdown pathways increases Kir2.1 steady state levels

HEK-KWGF cells were incubated with either 1 mM NH₄Cl, 10 μM chloroquine or 5 μg/mL leupeptin for 6 and 24 h, respectively. Subsequently, expression level of Kir2.1–GFP fusion protein was analyzed by Western blotting. As depicted in Fig. 1A, chloroquine rapidly increased Kir2.1–GFP expression levels within 6 h which was even more pronounced after 24 h. Leupeptin resulted in enhanced Kir2.1–GFP following 6 and 24 h of incubation. Only modest increased Kir2.1–GFP levels were observed by NH₄Cl application. Following the strong upregulation due to chloroquine or leupeptin treatment, an additional signal is observed at ~70 kDa. Since the fusion protein is detected by an antibody directed against the C-terminal fused GFP, this product most likely represents a protein product that results from N-terminal Kir2.1 cleavage. To further elucidate the kinetics of Kir2.1 upregulation, HEK-KWGF cells were treated with 10 μM chloroquine.
for 1, 2, 3, 4, 6 and 24 h, respectively (Fig. 1B). Increased expression levels of full-length Kir2.1–GFP were observed already after 1 h of treatment. Furthermore, the presumed ~70 kDa degradation product becomes detectable following 3 h of chloroquine treatment. These data indicate that the lysosomal degradation pathway is involved in Kir2.1–GFP breakdown, and that Kir2.1–GFP turnover takes place at a time scale of only a few hours.

**Lysosomal inhibition results in granular intracellular Kir2.1 accumulation**

Next, we assessed Kir2.1–GFP localization following application of the different inhibitors for 6 and 24 h. In non-treated cells, Kir2.1–GFP is mainly localized at the plasmamembrane. Chloroquine incubation leads to strong intracellular accumulation (Fig. 2A). Similar results were obtained using non-tagged Kir2.1 (Fig. 2B). Like for chloroquine, incubation with leupeptin or NH₄Cl results in intracellular accumulation of Kir2.1–GFP and appeared to increase plasmamembrane staining (Fig. 2A). We conclude that the strong increase in Kir2.1–GFP levels in chloroquine, and to a lesser extend leupeptin and NH₄Cl, treated cells substantially results from intracellular granular accumulation, presumably in late endosomes and/or lysosomes [23].

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**Fig. 1. Effect of lysosomal degradation inhibitors on Kir2.1–GFP steady state expression levels.** (A) Kir2.1–GFP protein expression levels in HEK-KWGF cells increase upon treatment with different lysosomal inhibitors. HEK-KWGF cell cultures were treated with 1 mM NH₄Cl, 10 μM chloroquine or 5 μg/mL leupeptin for 6 and 24 h, respectively. Kir2.1–GFP protein level in total cell lysates was detected using GFP antibody. (B) Kinetics of Kir2.1–GFP upregulation following treatment with 10 μM chloroquine. Cadherin expression levels were identical to total protein profiles as detected by Ponceau S staining prior to immunodetection (not shown), and are regarded as loading control using a pan-cadherin antibody.

**Fig. 2. Intracellular Kir2.1 accumulation upon treatment of HEK-KWGF or HEK-HsKir2.1 cells with lysosomal inhibitors.** (A) Cell cultures were treated as in Fig. 1. Subsequently, Kir2.1–GFP was detected in fixed cells using anti-GFP as primary and FITC-labeled secondary antibody. Scale bar represents 40 μm. (B) Intracellular accumulation of non-tagged Kir2.1 (green) upon treatment of HEK-HsKir2.1 cells with 10 μM chloroquine for 2 and 4 h, respectively. Kir2.1 was detected by anti-Kir2.1 antibody, nuclei are stained with DAPI (blue). Scale bar represents 20 μm. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

**Inhibition of lysosomal mediated Kir2.1 breakdown results in increased Iₖ₁ densities**

To see whether the increased protein levels as observed by Western blotting and intracellular accumulation would also result in enhanced functional Kir2.1–GFP expression at the plasmamembrane, HEK-KWGF cells treated with
chloroquine or leupeptin were analyzed for $I_{K1}$ current densities (Fig. 3A). As depicted in Fig. 3A and B, chloroquine significantly increased the inward component of $I_{K1}$ at membrane potentials between $-114$ and $-94$ mV, while both chloroquine and leupeptin significantly increased the outward component of $I_{K1}$ between membrane potentials of $-50$ and $-65$ mV (Fig. 3C). Furthermore, the reversal potential was not changed, which was reflected by a lack of difference in membrane potential ($-75.2 \pm 0.5$, $-75.5 \pm 0.5$ and $-74.9 \pm 0.6$ mV for control, chloroquine and leupeptin, respectively).

**Discussion**

In the current study we provide biochemical, immunofluorescence and electrophysiological evidence for involvement of the lysosomal degradation pathway in Kir2.1 breakdown. Results of Tong et al. [22] suggest that Kir2.1 channels enter the degradation pathway via clathrin mediated endocytosis. Whether all Kir2.1 channels are subsequently degraded via the lysosomal pathway is unknown. Alternatively, internalized channels may become targeted to proteasome mediated degradation or recycle to the plasmamembrane. Biochemical analysis displayed increased steady state expression levels within 1 h of treatment with lysosomal inhibitors (Fig. 1B). This implicates a relatively rapid turnover, within hours, of the Kir2.1 ion channel protein in HEK293 cells. Furthermore, lysosomal inhibition results in intracellular Kir2.1 protein accumulation as depicted in Fig. 2 within 2 h, however this does not necessarily imply a retarded internalization from the plasmamembrane. Apparently, our 24 h treatment with chloroquine and leupeptin results in saturation of the Kir2.1 degradation pathway which likely affects internalization capacity of the functional Kir2.1 channels, culminating in increased $I_{K1}$ densities. Preliminary studies indicated that NH$_4$Cl did not result in a significant increase in $I_{K1}$ densities, which is in line with the biochemical results. Furthermore, chloroquine and NH$_4$Cl display the same mechanism of action with respect to lysosomal degradation inhibition although with different efficiencies. In contrast to our results, transiently transfected Kir6.2 channels in COS cells do not display increased cell surface expression upon 6 or 12 h of chloroquine treatment [27]. This may be due to different methodology between their (chemiluminescence) and our (patch clamp) study to detect ion channel expression at the plasmamembrane. On the other hand, Kir6.2 channels may be degraded by a different pathway or multiple pathways, or at a different time scale. Finally, Kv1.5 voltage gated channel is degraded via the proteasomal pathway instead of the lysosomal pathway and blocking this degradation pathway results in increased $I_{Kur}$ current densities [28]. Obviously, no universal potassium ion channel protein degradation pathway seems to exist.

Chloroquine and leupeptin treatment yields a product that is approximately 10 kDa less in molecular weight than full-length Kir2.1–GFP. Since GFP is fused to the C-terminus of Kir2.1, we reason that this product is the result of N-terminal cleavage. It might have a much shorter half-life than full length protein and therefore only becomes detectable following maximal inhibition.

![Fig. 3. Quantification of functional membrane expression of Kir2.1 using the whole cell voltage clamp technique. (A) Diagram depicting the relation between steady state $I_{K1}$ current and test potential. Compared to controls, inward $I_{K1}$ currents are larger in chloroquine and leupeptin treated HEK293 cell cultures. Asterisk indicates a significant difference between control and chloroquine ($p < 0.05$). (B) Steady state conductance density negative to $E_K$ were significantly increased in chloroquine treated cultures, underscoring increased membrane localization of Kir2.1 channels ($p < 0.05$). (C) Detail of outward currents depicted in (A); legend as in (A). Both chloroquine and leupeptin treated cultures show significantly increased outward current (● and #, $p < 0.05$).](image-url)
of degradation. N-terminal cleavage might be the first step in Kir2.1 degradation.

Chloroquine has a long clinical history as an antimalarial drug and is associated with cardiac rhythm and conduction disturbances that can evolve in life-threatening arrhythmias [29]. In feline purkinje and ventricular cardiomyocytes, acute application of chloroquine results in block of various cardiac ion channels including the $I_{K_1}$ channel. [30]. In our studies, chloroquine acts at a completely different cellular level as a lysosomal inhibitor displaying opposite biological effects, i.e. an increase in $I_{K_1}$ current densities. Our electrophysiological measurements were performed in the absence of the drug and thereby exclude the acute effect of chloroquine on Kir2.1 based ion channels. These data indicate that chloroquine can display a dual effect on inward rectifier currents, directly by blocking the channel, and indirectly by inhibition of channel degradation. The resultant of long-term exposure to chloroquine in a complex system as the intact cardiomyocyte or heart, however, is difficult to predict and requires a comprehensive analysis of spatial and functional expression of Kir2.x isoforms.

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References

