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Evidence for proteasomal degradation of Kv1.5 channel protein

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Abstract

Background: The voltage-gated potassium channel Kv1.5 plays a critical role in the maintenance of the membrane potential. While protein degradation is one of the major mechanisms for the regulation of channel functions, little is known on the degradation mechanism of Kv1.5.

Methods and results: Kv1.5 was expressed in COS cells and its degradation, intracellular localization, and channel activities were assessed by pulse-chase analysis, immunofluorescence, and patch clamp techniques, respectively. Expressed Kv1.5 had a half-life time of ~6.7 h, which was prolonged by the proteasome inhibitors of MG132, ALLN, proteasomal inhibitor 1, or lactacystine, but not by a lysosomal inhibitor chloroquine. MG132 increased the protein level of Kv1.5, as well as the level of its ubiquitinated form in a dose-dependent manner. Similar effects of MG132 on endogenous Kv1.5 were seen in cultured rat atrial cells. Within a cell, Kv1.5 was mainly localized in both the endoplasmic reticulum and Golgi apparatus. MG132 increased the immunoreactivity of Kv1.5 in these compartments and also increased Ik_{ur} currents through the cell-surface Kv1.5. Pretreatment with either brefeldin A or colchicine abolished MG132-induced increase in Ik_{ur} currents.

Conclusion: Kv1.5 is degraded by the proteasome. The inhibition of the proteasome increased Ik_{ur} currents secondary to stabilization of the channel protein in the endoplasmic reticulum/Golgi apparatus.

Keywords: Kv1.5; Ubiquitin; Proteasome; MG132; Ik_{ur}

The voltage-gated potassium channel Kv1.5 confers an ultrarapid delayed rectifier current (Ik_{ur}) and plays an important role in the maintenance of the membrane potential [1]. Cardiac Kv1.5 is of particular clinical importance as a target of various antiarrhythmic drugs [2]. The channel activity can be controlled by the protein level expressed in an individual cell which in turn is controlled both by transcriptional and post-transcriptional mechanisms [3]. Of the various post-transcriptional control mechanisms,

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regulation of protein degradation may be particularly relevant for proteins with a relatively short half-life time [4]. Intracellular proteolysis of channel proteins can take place either in the endosome/lysosome or in the cytosol by the proteasome [5]. It has been reported that channel proteins with relatively short half-life times, such as an epithelial Na^+ channel (eNaC) [6], a cystic fibrosis transmembrane regulator (CFTR) [7], a gap junction channel connexin 43 [8], and human ether-a-go-go related gene (HERG) channels [9], are degraded through the ubiquitin–proteasome pathway. HERG belongs to a large superfamily of voltage-gated potassium channels [9] including Kv1.1 and KvLQT1. Although it has been reported that the Kv1.5 channel, a member of the superfamily of voltage-gated potassium channel, had a relatively short half-life [4], it is not known whether it is degraded through the ubiquitin–proteasome pathway. Following confirmation of the proteasomal degradation of this channel protein, we examined whether the inhibition of the proteasome activity caused any changes in the intracellular localization and/or channel activity of Kv1.5 proteins.

Methods

Plasmids and expression. An expression construct pRC/Kv1.5-FLAG was engineered by ligating an oligonucleotide encoding a FLAG epitope to the carboxy terminus of rat Kv1.5 cDNA. COS cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL)/10% fetal bovine serum at 37 °C in a 5% CO_2 incubator. Cells were transfected by using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. 48 h after transfection, cells were subjected to assays. Brefeldin A or colchicine was added to the culture medium 12 h after the transfection. Proteasome inhibitors were applied 36 h after transfection. All drugs, except for MG132, were dissolved in a buffer. MG132 was dissolved in DMSO. The final concentration of DMSO in the culture or reaction medium was equal to or less than 0.01% v/v.

Western blotting and immunoprecipitation. Cells were scraped into lysis buffer (PBS/1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstain, and 1 mM phenylmethylsulfonyl fluoride), lysed by sonication, and insoluble materials were removed by centrifugation. Protein concentrations were determined with a BCA protein assay kit (Pierce). Ten micrograms protein was separated on SDS–PAGE and electrotransferred to a PVDF membrane. Membranes were proved with antibodies against FLAG (1:1000, Cosmo Bio), GFP (1:4000, Molecular Probes), Kv1.5 (1:100, Alomone Lab), ubiquitin (1:1000, MBL) or β-actin (1:5000, Oncogene) and were developed using an ECL system. Immunoprecipitation was carried out in PBS/1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 1 mM EDTA, and protease inhibitors for 2 h at 4 °C. Immunocomplexes were collected with protein G–agarose (Pharmacia) and bound proteins were analyzed by SDS–PAGE followed by immunoblotting.

Pulse-chase analysis. Cells were pulse-labeled for 2 h in methionine-free DMEM supplemented with [^{35}S]methionine (3.7 μCi/ml: Amersham) and then chased in DMEM supplemented with 1 mM methionine. Where indicated, proteasome inhibitors were included both in pulse and chase media. Anti-FLAG precipitation was carried out as described above and bound proteins were analyzed by SDS–PAGE followed by autoradiography. The band intensities were quantified using an NIH image software. The decay constant (k) was estimated by fitting first-order decay curves to the form $y = e^{-kt}$, using SigmaPlot (Jandel Scientific). A half-life time was calculated using a formula $t_{1/2} = 0.693/k$ [10].

Immunofluorescence. Cells were transfected with pRC/Kv1.5-FLAG and Golgi-EYFP or endosome-EGFP constructs (Clontech). Anti-FLAG staining of fixed cells was performed as described [11] by using Texas red-conjugated anti-mouse IgG as a secondary antibody and images were collected with a Bio-Rad MRC1024 confocal microscope. To quantify the immunoreactivity, we measured the levels of Texas red fluorescence (which emanated from the secondary antibody) and EYFP fluorescence (which emanated from Golgi-EYFP) in lysates of stained cells using a fluorophotometer and determined the ratio of Texas red/EYFP fluorescence.

Primary culture of rat atrial myocytes. Male Wistar rats weighing 120 to 180 g were anesthetized with pentobarbital and hearts were quickly removed. Atrial cells were isolated by enzymatic digestion and cultured for 12 h in the presence or absence of indicated drugs. Anti-Kv1.5 blotting of cell lysates and anti-ubiquitin blotting of anti-Kv1.5 precipitates were performed as described above.

Electrophysiological recordings. Cells were co-transfected with pRC/Kv1.5-FLAG and pEGFP. Transfected cells were visualized by EGFP fluorescence and subjected to whole-cell voltage clamp experiments. Briefly, currents were elicited every 6 s from a holding potential of −60 to +60 mV by consecutive steps every 20 mV for 500 ms. Ik_{ur} currents were defined as the difference between the absence and presence of 1 mM 4-aminopyridine (4-AP).

Results

Kv1.5-FLAG was ubiquitinated and degraded by the proteasome

Kv1.5-FLAG expressed in COS cells was detected by anti-FLAG Western blotting at ~80 kDa, a predicted size of full-length Kv1.5-FLAG. Treatment with MG132 (50 μM for 12 h) increased the density of this band (Fig. 1A), suggesting MG132-induced stabilization of Kv1.5-FLAG. The same treatment did not affect the level of co-expressed GFP (Fig. 1A). Kv1.5-FLAG was recovered in the detergent-soluble fraction regardless of MG132 treatment, excluding drug-induced changes in protein solubility (Fig. 1B). MG 132 effects were concentration-dependent with an EC_{50} value of ~4.3 nM (Fig. 1C). Immunoprecipitation experiments showed that MG132 treatment increased levels of ubiquitinated Kv1.5-FLAG (Fig. 1D).

To confirm MG132-induced stabilization of Kv1.5-FLAG, we determined a half-life time ($t_{1/2}$) of expressed proteins by pulse-chase analyses. Kv1.5-FLAG had a $t_{1/2}$ of 6.7 ± 1.2 h ($n = 4$), and this value was prolonged to 20.2 ± 3.4 h ($n = 4$) in cells treated with MG132 (Fig. 1E). Both proteasome inhibitors lactacystine (25 μM) and proteasome inhibitor 1 (100 μM) had similar effects, whereas a lysosome inhibitor chloroquine (2 mM) had no effect (data not shown). There were similar levels of ^{35}S-labeled proteins between drug-treated and -untreated cells at the end of the pulse period (time 0 point in Fig. 1E), excluding an effect of MG132 on protein synthesis.

Increased levels of Kv1.5 in primary-cultured atrial myocytes treated with MG132

We tested whether a similar stabilizing effect was observed in primary-cultured atrial myocytes, which express a relatively high level of Kv1.5 [12]. MG132 treatment (50 μM for 12 h) increased the level of atrial Kv1.5. Anti-ubiquitin blotting of anti-Kv1.5 precipitates revealed

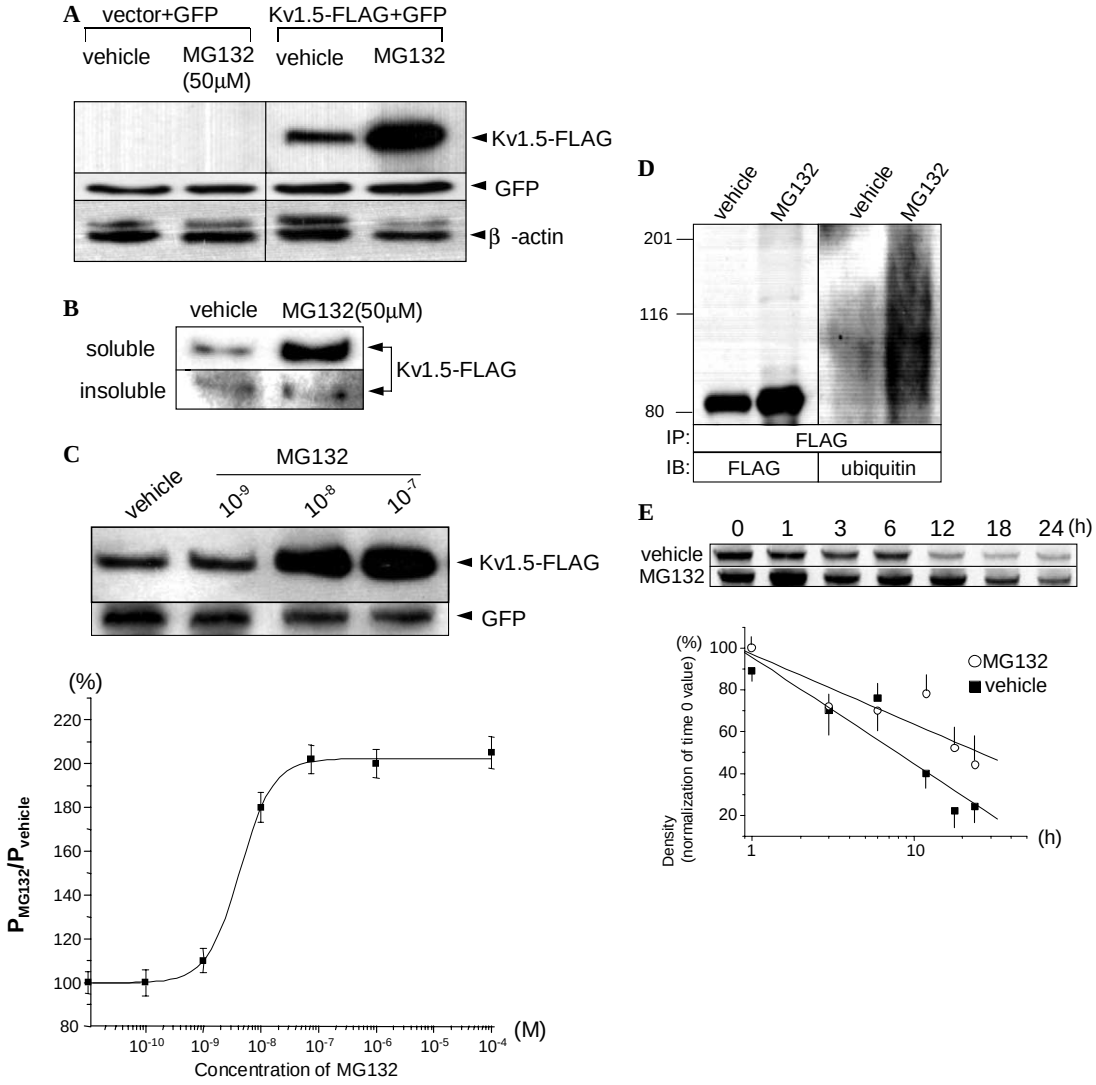

Fig. 1. Effects of MG132 on Kv1.5-FLAG expressed in COS cells. Cells were transfected with Kv1.5-FLAG and GFP constructs and treated with MG132 (50 μM) or vehicle (0.01% DMSO) for 12 h. (A) Effects on protein levels. Cell lysates were subjected to Western blotting with antibodies against indicated proteins. (B) Effects on protein solubility. Soluble and insoluble fractions of cell lysates were subjected to anti-FLAG Western blotting. (C) Dose-dependent effects of MG132 on the level of Kv1.5 protein. Cells were transfected with Kv1.5-FLAG and GFP constructs, and treated with MG132 in a dose-dependent manner. Concentration–response curve were constructed from four independent experiments covering the entire concentration range by fitting Hill equation of the form shown in Eq. (1) to mean value ± SE

$$P_{\mathrm{MG132}}/P_{\mathrm{vehicle}} = 1/(1 + (D/K_{\mathrm{d}})^{n}), \quad (1)$$

where P indicates the intensity of Kv1.5-FLAG protein band, D is the drug concentration, n is the Hill coefficient, and K_{d} is the half-maximal concentration of MG132 to increase Kv1.5-FLAG protein. (D) Effects on ubiquitination. Anti-FLAG immunoprecipitates (IP) were subjected to immunoblotting (IB) with indicated antibodies. Molecular weights are indicated on the left (kDa). All these experiments were repeated five times with similar results. (E) Effects on protein stability. Cells were pulse-labeled with [^{35}S]methionine and chased for indicated times. Both pulse and chase media contained MG132 or vehicle. Shown are the representative autoradiograph (upper) and semi-log plots of decay curves (lower).

increased levels of ubiquitinated Kv1.5 in cells treated with MG132 (Fig. 2).

Kv1.5-FLAG was mainly localized in the endoplasmic reticulum and Golgi apparatus

Next, we examined intracellular localization of Kv1.5-FLAG by immunofluorescence. Anti-FLAG staining of transfected COS cells revealed localization of Kv1.5-FLAG in a reticular pattern throughout the cytosol, obviously in the endoplasmic reticulum (ER). Kv1.5-FLAG was also localized in the Golgi apparatus as shown by its co-localization with Golgi-EYFP (Fig. 3A). We found no co-localization of Kv1.5-FLAG and Endosome-EGFP (data not shown). Anti-Kv1.5 staining of COS cells expressing Kv1.5 showed similar distribution (data not shown), excluding a potential effect of the FLAG epitope on the intracellular localization.

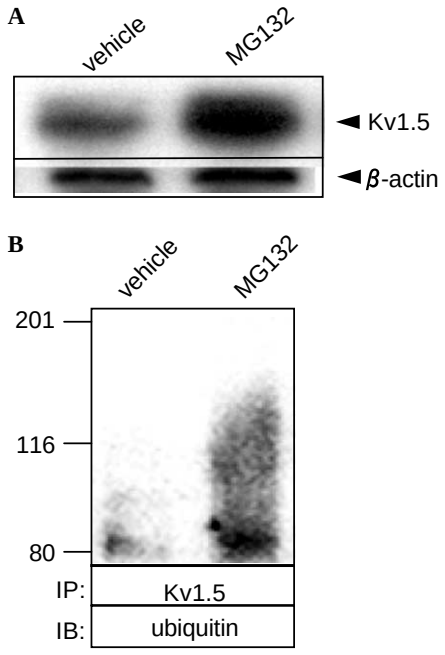

Fig. 2. Effects of MG132 on Kv1.5 protein levels in primary-cultured atrial myocytes. Cells were cultured for 12 h in the absence or presence of MG132 (50 μM). (A) Effects on the protein levels. Cell lysates were subjected to the Western blotting with indicated antibodies. (B) Effects on the ubiquitination. Anti-Kv1.5 IP products were subjected to IB with indicated antibody. Molecular weights are indicated on the right (kDa). Shown are the representative results obtained in three independent experiments.

MG132 treatment (50 μM for 12 h) apparently increased the Kv1.5-FLAG immunoreactivity both in the ER and Golgi apparatus (Fig. 3A). To confirm this increase, we quantified Texas red fluorescence (which emanated from the secondary antibody bound to anti-FLAG) and EYFP fluorescence (which emanated from co-expressed Golgi-EYFP) in cell lysates. The MG132 treatment caused a significant increase in the Texas red/EYFP fluorescence ratio (Fig. 3B). MG132 treatment caused no significant decrease in cell viability as assessed by an MTT conversion assay (data not shown), excluding a cytotoxic effect of this compound.

MG132-induced increase of Ik_{ur} currents through cell-surface Kv1.5-FLAG

To examine whether MG132 also altered the levels of cell-surface Kv1.5-FLAG, we examined Ik_{ur} currents. Depolarizing test pulses elicited outward currents in cells expressing Kv1.5-FLAG and these currents were partially blocked by 4-AP. This 4-AP-sensitive component was considered to represent Ik_{ur} currents [4]. Regardless of MG132 treatment, we found no 4-AP-sensitive currents in cells transfected with an empty vector plasmid (data not shown), indicating that the observed Ik_{ur} currents were totally derived from expressed Kv1.5-FLAG. The kinetics of Ik_{ur} currents through Kv1.5-FLAG were indistinguishable from those through Kv1.5, indicating no interference of the FLAG tag with the channel

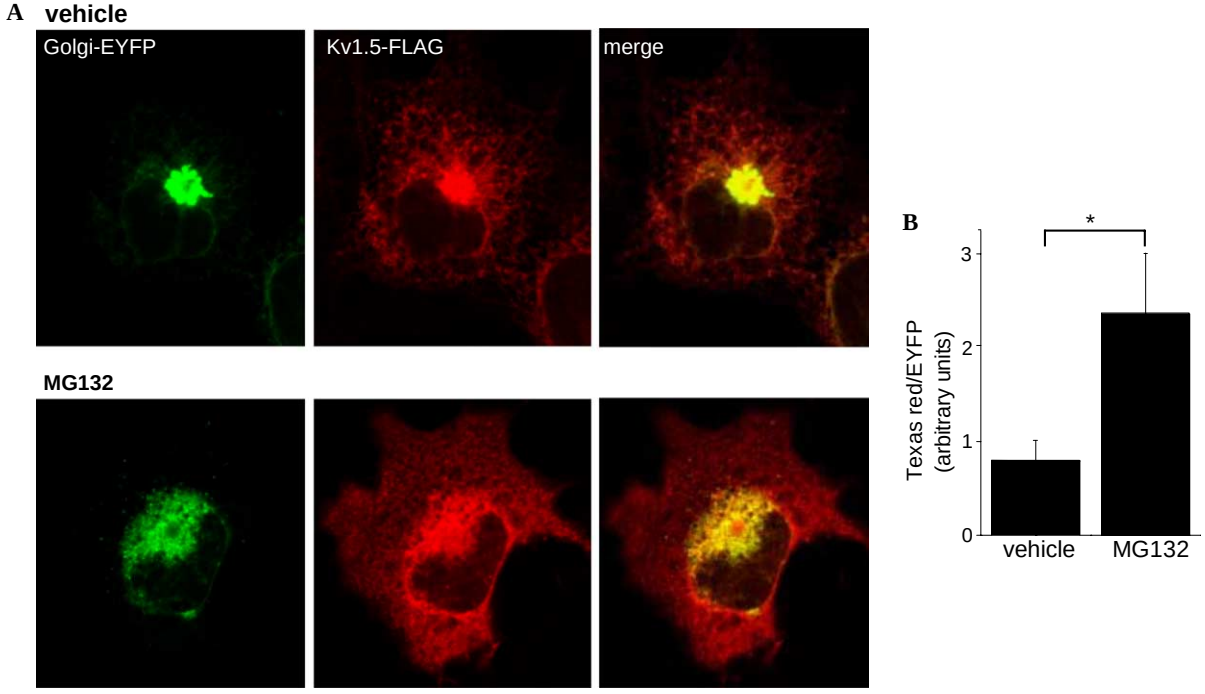

Fig. 3. Immunofluorescence of Kv1.5-FLAG. (A) Cells were transfected with Kv1.5-FLAG and Golgi-EYFP constructs, treated with MG132 (50 μM) or vehicle for 12 h, fixed, and stained with anti-FLAG. Shown are the representative images obtained with a confocal microscope. (B) Quantification of anti-FLAG immunoreactivity. Fluorescence of Texas red and EYFP in cell lysates was determined by a fluorophotometer. Shown is the ratio of Texas red/EYFP fluorescence. Each bar represents mean ± SEM of 15 determinations. *$p < 0.05$ significantly differences from each other (one-way ANOVA).

activity (data not shown). MG132 treatment (50 μM for 12 h) obviously increased Ik_{ur} currents (Fig. 4A). Current-voltage relationships showed that the MG132 treatment increased the amplitude of Ik_{ur} currents in a range from +20 to +60 mV but did not affect the threshold potential (Fig. 4B).

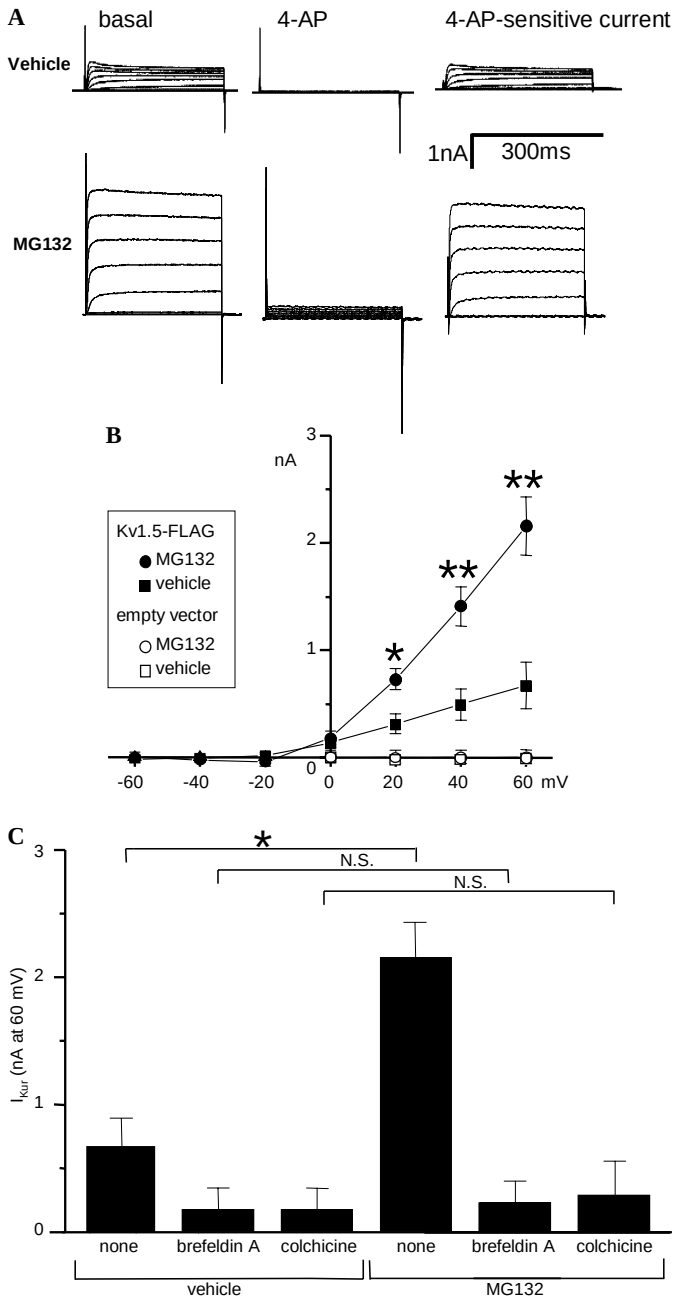

Fig. 4. Effects of MG132 on Ik_{ur} currents in COS cells expressing Kv1.5-FLAG. (A) Representative current traces of cells expressing Kv1.5-FLAG and treated with MG132 (50 μM) or vehicle for 12 h. The currents were elicited by test potentials of each +20 mV for 500 ms from a holding potential of −60 mV. Right panels show 4-AP-sensitive currents defined as the difference between the absence and presence of 4-AP. (B) Current–voltage relationships of 4-AP-sensitive currents. Each point represents mean ± SEM of 6–17 determinations. (C) Effects of protein transport-blockers. Transfected cells were treated with brefeldin A or colchicine (both at 5 μM for 36 h) and with MG132 or vehicle before determination of 4-AP-sensitive currents. Each bar represents mean ± SEM of 8–17 determinations. $*p < 0.05$, $**p < 0.01$ significantly different from the control values, N.S. not significantly different (one-way ANOVA).

Since our immunofluorescence experiments showed a significant increase in Kv1.5-FLAG proteins in the ER and Golgi apparatus of the cells treated with MG132, it was likely that the MG132-induced increase of Ik_{ur} currents was secondary to stabilization of this protein in these compartments. To substantiate this notion, we tested effects of brefeldin A and colchicine on Ik_{ur} currents. Brefeldin A inhibits ADP-ribosylation factor-dependent protein transport in the Golgi apparatus [13] and colchicine inhibits microtubule-dependent protein transport [14]. Treatment with these drugs (both at 50 μM for 12 h) decreased the basal level of Ik_{ur} currents and abolished the MG132-induced increase (Fig. 4C). Neither drugs caused any changes in Ik_{ur} currents when applied immediately before the recording (data not shown).

Discussion

When expressed in COS cells, Kv1.5-FLAG had a relatively short half-life time and a part of the protein was ubiquitinated. A proteasome inhibitor MG132 prolonged the half-life time and increased the steady state levels of the protein as well as its ubiquitinated form (Fig. 1). Similar effects were observed with other proteasome inhibitors lactacystine and proteasome inhibitor 1 but not with a lysosomal inhibitor chloroqine. This stabilization of Kv1.5-FLAG by proteasomal inhibition has been observed in the primary cultured atrial myocytes (Fig. 2). Together with the localization of expressed proteins in the ER/Golgi apparatus but not in the endosome (Fig. 3), these results strongly argue that Kv1.5-FLAG was degraded in the cytosol by the proteasome but not in the endosome/lysosome.

It is well accepted that immature channel proteins localized in the ER/Golgi can be degraded by the proteasome. On the other hand, connexin 43 [8] on the cell surface undergoes ubiquitination and proteasomal degradation, indicating that mature proteins on the cell surface can also be a target of proteasomal degradation [15]. Of these candidate sites, the main site of MG132 action on Kv1.5 appeared to be the ER/Golgi apparatus, because this drug increased the levels of Kv1.5 in these compartments, without affecting the localization pattern (Fig. 3).

Inhibition of the ubiquitin proteasome system increased the number of ENaC channels at the plasma membrane [6]. In contrast, inhibition of the proteasome did not result in an increase of the mature forms of CFTR [7] or HERG [9]. We found in the present study that MG132 increased cell-surface-expression of mature Kv1.5, as evidenced by an increase of Ik_{ur} currents, is most likely secondary to the stabilization of the channel proteins in the ER/Golgi apparatus. This notion is consistent with the effects of brefeldine A and colchicine, both of which abolished the effect

of MG132 on Ik_{ur} currents, presumably by inhibiting intracellular transport of mature proteins to the cell surface (Fig. 4).

Proteasomal degradation of immature proteins is a part of the ER quality control system and the pathological implication of this system has been well established for eNaC, CFTR, and HERG: accelerated degradation of mutants of these channel proteins results in inherited diseases, Liddle syndrome [6], cystic fibrosis [7], and long QT syndrome [9], respectively. Accelerated degradation of connexin 43 has also been implicated in the electrical disturbance of the cardiac failure [16]. Besides its role in the quality control, proteasomal degradation may also be implicated in the quantity control under physiological conditions; levels of cell-surface HERG can alter cell excitability and levels of connexin 43 can affect cell synchronization [8]. It is currently unknown whether proteasomal degradation of Kv1.5 is a simply part of the quality control system of this channel protein or rather implicated in the quantity control under physiological and/or pathological conditions. In this context, the electrical remodeling of the heart in the patients with atrial fibrillation is associated with a decrease of atrial Kv1.5 channels [17]. It is the subject for a future study whether this decrease was due to accelerated degradation or not.

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